In vitro synergy of tigecycline against *Burkholderia cepacia* complex and other multi-resistant, non-fermentative, Gram negative bacteria from cystic fibrosis patients

John Kenning, PGCE, BSc (Hons), MSc

Specialist Biomedical Scientist

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School of Pharmacy and Biomedical Sciences
Faculty of Science
University of Portsmouth

Aegrotos sanate, leprosos purgate:

Dono accepistis, dono date.

Mathew 10:8

Abstract

Options for treating *Burkholderia cepacia* complex (Bcc) and other multi-resistant Gram negative bacilli isolated from people with cystic fibrosis (CF) are limited. We assessed the *in-vitro* activity of tigecycline and eleven other antimicrobial agents against a collection of these organisms. The collection comprised 128 isolates of CF-associated Gram negative bacilli (31 *Burkholderia multivorans*, 16 *Burkholderia cenocepacia*, 4 other members of the *Burkholderia* species, 47 *Stenotrophomonas maltophilia*, 20 *Achromobacter xylosoxidans*, and 10 other miscellaneous CF-associated Gram negative bacilli. Minimum inhibitory concentrations (MIC) of tigecycline and eleven other antimicrobials for each isolate were determined using E-test. Synergy between tigecycline and each of eight other antimicrobials was determined using an E-test overlay method. The epidemiological spread of organisms indicated that the Leeds Teaching Hospitals NHS Trust (LTHT) infection control policies have had a measure of success and our work followed the pattern of many other CF units. Tigecycline showed poor *in-vitro* activity versus all members of the Bcc, with only 13% and 3% of *B. cenocepacia* and *B. multivorans* susceptible, respectively. Conversely minocycline showed good activity against these species, with 94% and 91% of isolates being susceptible. Tigecycline showed good activity against *A. xyloxidans* and *S. maltophilia* with 85% and 77% of isolates being susceptible, respectively. Tigecycline in combination with other agents mostly resulted in indifference. Although the *in-vitro* activity of tigecycline is variable, we reviewed the potential and future clinical impact of this study and the likely issues for further study. Whilst the relationship between synergy/MIC testing and clinical success remains unclear, there are a number of promising developments and ideas that may clarify this situation – further studies are warranted.

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Table of Contents

List of abbreviations

ASL	Airway surface liquid
Bcc	*Burkholderia cepacia* complex
BSAC	British Society for Antimicrobial Chemotherapy
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CLED	Cystine, lactose, electrolyte deficient agar
CLSI	Clinical laboratory standards institute
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
ESBL	Extended spectrum β-lactamase
ESR	Erythrocyte sedimentation rate
FEV1	Forced expiratory volume in 1 second
FIC	Fractional inhibitory concentration
(∑FIC)	(Summation fractional inhibitory concentration)
GNB	Gram negative bacillus(i)
LTHT	Leeds Teaching Hospitals NHS Trust
MCBT	Multiple combination bactericidal testing
MIC	Minimum inhibitory concentration
PBP	Penicillin binding protein
PCD	Primary ciliary dyskinesia
PCL	Pericilliary liquid layer
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
RFLP	Restriction fragment length polymorphism
SCV	Small colony variant

List of Figures

List of Tables

Chapter 1

1.0 Introduction

Cystic fibrosis (CF) is an autosomal recessive disease (Belkin *et al*, 2006), and is one of the most common of all lethal genetically inherited diseases with associated morbidity. It affects around 1/2000–1/3000 live births in the Western world, though estimates vary (Walters & Mehta, 2007), and is particularly prevalent in the Caucasian population (O'Sullivan & Freedman, 2009).

The disease occurs in approximately 1/3000 white Americans, 1/400-10,000 Latin Americans and 1/15,000-20,000 African Americans. It is very unusual to encounter CF in either Africa or Asia – for example 1/350,000 in Japan are afflicted (O'Sullivan & Freedman, 2009). The most common occurrence of CF appears to be in populations of northern European descent (1/3000 live births).

CF is characterised by a significant reduction in lung function – obstructive lung disease and chronic endobronchial infection and colonisation, along with other non pulmonary symptoms. It is a disease that has seen major developments over the last 10-20 years both in terms of therapy/research and life expectancy. In 2006, the median life expectancy in the USA was 33 years (Belkin *et al*, 2006), whereas the predicted life expectancy in the UK has risen from 31 years, to 50 years (Dodge *et al*, 2007). This rise is primarily caused by the development and use of intensive antimicrobial therapy over the past 20 years along with updated and thorough infection control practices (Millar *et al*, 2009, Saiman & Siegel, 2004).

It is estimated that approximately 90% of all CF patients die as a result of end stage obstructive lung disease – making CF one of the top three indications for lung

transplantation (Belkin *et al*, 2006). Even with current transplantation criteria, however, approximately 30% of patients will die before lung transplantation is offered. Lung transplantation is not always an option, and this is particularly the case with patients who have been colonised by resistant bacteria such as *Burkholderia cepacia complex* (Bcc) organisms (Liou *et al*, 2005). CF patients are susceptible to infection and colonization by a number of organisms, the most common being *Pseudomonas aeruginosa* – which affects over 90% of patients at some point during their lives (Lyczak *et al*, 2002). Other frequently isolated organisms include *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*. Bcc are a major cause of mortality and morbidity via sepsis, rapid deterioration and necrotizing pneumonia – often leading to death by the so called "Cepacia syndrome" (Isles *et al*, 1984; Aaron *et al*, 2000).

1.1 Pathophysiology of cystic fibrosis and the cystic fibrosis transmembrane regulator gene

Chronic bacterial infection of the airways is generally associated with blockage/obstruction. In many patients, this can be caused by tumours or foreign bodies. In CF patients, however, it is more usually the result of mucus plugs, adhesion and plaques. Similar obstructions also contribute to other conditions such as chronic obstructive pulmonary disease (COPD). Observations from many studies, including murine models, suggest that mucus clearance is the dominant form of innate defence. This is severely compromised in CF patients. CF, when compared with conditions such as primary ciliary dyskinesia (PCD) results in more severe airway destruction and infection – indicating that mucus clearance may be more important than the action of the cilia (Randell & Boucher, 2006).

In normal patients, the carefully co-ordinated system of epithelial water and ion transport, mucin secretion, and cilia action are responsible for airway clearance and maintenance. Water transport across the apical plasma membrane of airway epithelial cells is regulated by

chloride ion export through cystic fibrosis transmembrane regulator (CFTR) gene regulation – thus maintaining the airway surface liquid (ASL) status (Randell & Boucher, 2006).

The close cell environment in the lungs has increasingly been recognised as one of the most important factors in airway clearance. Two separate layers exist that together comprise the ASL. The first is an overlying transported mucus layer. Underneath this is a distinct close cell layer – the pericilliary layer (PCL). Previous studies have found that the addition of liquid to this layer (usually brought about by medical conditions such as pseudohypoaldosteronism) can enhance the activity of cilia and thus the mucus clearance of the ASL (Randell & Boucher, 2006). The mucus layer of the ASL consists of high-molecular weight mucin dimers and trimers that interact with globular proteins. It is thought that non-mucin proteins act as a cross link whilst also providing defence against pathogens and foreign bodies. The near cell surface liquid of the PCL acts to preserve the architecture and movement of the cilia, whilst also allowing the movement of water and preventing foreign bodies such as microscopic particles from actually touching the cellular surfaces (Mall *et al*, 2004).

CF is brought about by a mutation in the CFTR protein producing gene. There are approximately 1500 CFTR mutations that have been identified, though the function of only a small number of these has been ascertained (O'Sullivan & Freedman, 2009) and CFTR is often classified as either absent, deficient or dysfunctional (David *et al*, 2009). The mutations in the CFTR gene vary with geography – *Phe508del*, for example predominates in Northwest Europe, whereas *Trp1282X* dominates in Israel (O'Sullivan and Freedman, 2009). The CFTR gene has many regulatory roles including the inhibition of sodium transport, regulation of the outwardly rectifying chloride channel, regulation of ATP channels, regulation of intracellular vesicle transport, acidification of intracellular organelles and inhibition of endogenous calcium activated chloride channels, although its main role is to act as a chloride channel (O'Sullivan and Freedman, 2009). It is these two main functions

of CFTR that contribute to the pathophysiology of the disease. It is thought that the development of airway-surface liquid depletion is responsible for the collapse of cilliary function and the resultant loss of mucus clearance (Ratjen, 2009). The actual process is thought to come about by the forcible interaction between cell surface mucins and the viscous, adhesive mucus layer. The result of this PCL collapse, is the build up of mucus adhesion, the formation of plaques, and plugs of concentrated mucus (Mall *et al*, 2004).

It would appear that the severity of the disease in many patients may be linked to the relative functionality of the CFTR protein and the effect of the mutations upon it. Five classes have been identified, each of which may alter the presentation; for example, class I – three mutations in the CFTR gene are linked with pancreatic insufficiency. It can be difficult to predict the pulmonary implications of each class due to the wide range of differing genetic backgrounds of patients. Clinical presentation at CF clinics can and does vary greatly (David *et al*, 2009).

1.1.1 Pathological mechanisms

The airway epithelia regulate the properties of a thin layer of liquid – known as the ASL and it is this layer that is affected along with the cilia in CF patients. The mechanisms that lead to the symptoms and pathology of CF are unclear and there has been much argument in the literature (Tarran *et al*, 2001). The two main hypotheses, however, relate to the salt concentration in the airway surface, or the volume of the ASL. A number of different causative mechanisms have been suggested and these are summarised below.

1.1.2 The "high salt hypothesis"

It is suggested that the deregulation of the chloride and sodium transport systems may lead to increased concentrations of these chemicals in the PCL (in a normal patient, the NaCl concentration is low at < 50 mM). The high salt concentration and lack of regulation in CF patients may cause a reduction in the function of molecules that are naturally produced to

regulate or clear colonising bacteria – such as β-defensin 1. This means that bacteria are able to colonise the lungs in the manner presented by many CF patients, as defensins have been demonstrated to operate inefficiently in salt concentrations > 100 mM (Goldman *et al*, 1997).

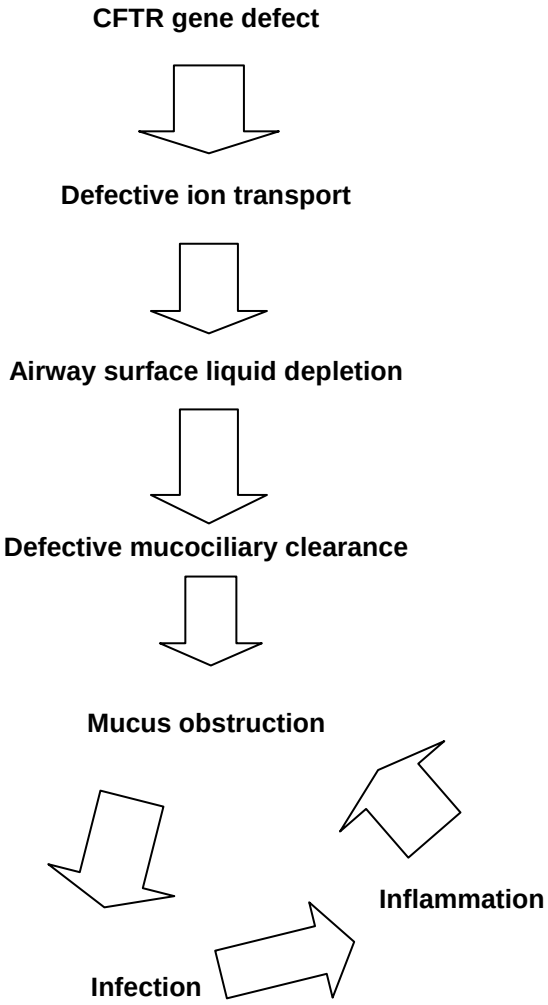

Fig 1.1: Pathophysiology in CF lung disease (adapted from Ratjen, 2009)

1.1.3 The "low volume hypothesis"

This postulates that the CFTR gene is unable to continue regulating the epithelial salt channels. This results in excessive absorption of sodium and water - leading to dehydration of the lungs and associated airways – low ASL. The water that should be present in the lungs acts both as a medium to enhance absorption of oxygen, but also as a lubricant. This

loss of lubrication may result in the build up of mucus plaques on the epithelium resulting in small "hypoxic niches" that are able to provide a growth environment for colonising bacteria – particularly *Pseudomonas aeruginosa* (O'Sullivan & Freedman, 2009). It is also suggested that this lack of lubrication will allow the mucus to form a compressing layer on the cilia of the lungs (the PCL) – thus reducing their function. This results in the patient being unable to clear mucus from the airway. The loss of the chloride efflux system prevents the lungs from replacing and regulating this loss of water (Tarran *et al*, 2001). Whilst the host inflammatory response has also been suggested as a single reason for CF pathology, it has also been suggested that the low volume ASL may result in higher concentrations of inflammatory mediators. This would induce an inflammatory process with "self reinforcing pathologic sequelae" (Belkin *et al*, 2006). A study in 1998 by Hirotoshi *et al*, found that this reduction in ASL was the dominant factor in CF patients, and in this case, they were unable to find any evidence that the ASL was hypotonic or that salt concentrations differed significantly from those of normal patients. It has been suggested, however, that the ion transport theory may be more consistent with the pathogenesis of "other organ-level phenotypes of CF" (Hirotoshi *et al*, 1998). It remains unclear which of the effects presented here are dominant in the CF lung and as stated by Hirotoshi *et al* (1998), this may differ between patients and depend upon the level of CFTR inhibition.

1.1.4 "The CFTR binding hypothesis"

This suggests that where functional CFTR is present, any colonising bacteria would preferentially bind to it. The result is a self limiting, short lived host immune response – clearing any colonisation. In CF patients, this response is not present, and it is thought that an increase in asialo-GM1 in apical cell membranes allows increased binding of pathogens to the airway epithelium; these two factors together result in the rapid colonisation of the airways (O'Sullivan & Freedman, 2009).

1.1.5 Host inflammation

It appears that the host inflammatory response is present regardless of the infective state of the patient (O'Sullivan & Freedman, 2009). Mediators of inflammation such as TNFα, interleukin 6 and 8 and arachidonic acid metabolites have been found in CF patients. Additionally, a lack of anti-inflammatory substances such as decohexaenoic acid, lipoxin and interleukin 10 indicate that in these patients, the immune response is free to run unabated. These pro-inflammatory markers have been found in children as young as four when bronchial alveolar lavages were tested (O'Sullivan & Freedman, 2009).

The low volume hypothesis and high salt hypothesis are mutually exclusive in their properties *in-vivo*. The suggestions implicating that in a high salt concentration environment model, the ASL should remain normal, whereas, in a low ASL environment, the NaCl concentration remains normal. This makes it difficult to suggest a therapeutic strategy to treat the initiating events of CF (Tarran *et al*, 2001).

1.2 Presenting symptoms of cystic fibrosis

The clinical picture and presenting symptoms of CF vary greatly between patients whilst showing some overlap (O'Sullivan & Freedman, 2009).

The earliest symptoms in neonates include any of a combination of meconium ileus (approximately 15%), protracted jaundice, abdominal or scrotal calcifications and intestinal atresia. Meconium ileus is an obstructive bowel condition that affects both the small and large bowel. Clubbing of fingers and toes is often seen along with vitamin D deficiency. The resulting osteoporosis generally begins in childhood but is not often detected until later in life. Given the poor outlook and risk of malnutrition of CF patients, it is not surprising that, even in well nourished patients, bone formation is unable to keep up with bone resorption (O'Sullivan & Freedman, 2009).

Pancreatic insufficiency is seen in the infant/neonate and presents as abdominal bloating, failure to thrive and steatorrhoea. Malnutrition resulting from these conditions was a major cause of death in the early years of CF medicine when the condition was new to medical science (Anderson, 1938). Since 1938, pancreatic enzyme replacement therapy has been introduced, virtually ending these deaths. This does remain an issue, however, and must be monitored with calorific intake and fat soluble vitamins being of uttermost importance (O'Sullivan & Freedman, 2009).

Table 1.1: Signs and symptoms of cystic fibrosis (adapted from O'Sullivan & Freedman 2009)

General (any age)	Family History of cystic fibrosis	Productive cough
	Salty tasting skin	Isolation of airway *Pseudomonas aeruginosa*
	Clubbing of fingers and toes	Hypochloraemic matabolic alkalosis
Neonatal	Meconium ileus	Intestinal atresia
	Protracted jaundice	
	Abdominal/scrotal calcifications	
Infancy	*S.aureus* pneumonia	Chronic diarrhoea
	Cholestasis	Failure to thrive
	Abdominal distention	Infiltrates on chest radiographs
Childhood	Anasarca or hypoproteinaemia	Idiopathic intracranial hyportension (Vit A deficiency)
	Steatorrhoea	Haemolytic anaemia (Vit E deficiency)
	Rectal prolapse	Chronic pansinusitis/nasal polyposis
	Liver disease	distal intestinal obstruction syndrome
		idiopathic recurrent or chronic pancreatitis
Adolescence Adulthood	Bronchiectasis	Azoospermia (abscence of vas deferens)
	Haemoptysis	Idiopathic recurrent pancreatitis
	Delayed puberty	Allergic bronchopulmonary aspergillosis
	Portal hypertension	Chronic pansinusitis or nasal polyposis

Pancreatic dysfunction is caused by the obstruction of the intrapancreatic ducts and the resulting autolysis of the pancreas – replacing pancreatic tissue with fat. The resulting CFRD – CF-related diabetes mellitus, is a unique disease resulting from this damage. It is generally related to glucagon deficiency, liver dysfunction, raised energy expenditure and poor glucose metabolism. Decreased intestinal transit time and increased pulmonary workload (difficulty breathing) are also common. CFRD is reported in approximately 30%

of patients over the age of twenty five (O'Sullivan & Freedman, 2009). Of the people who suffer with CFRD, females appear to have a higher mortality (O'Riordan *et al*, 2008). Because of the increased risk to patients with CFRD, regular screening is recommended (by way of an annual glucose tolerance test) to those patients over the age of ten years. Blockages of various types appear to affect CF patients throughout their lives. In children, the obstruction of the intrahepatic bile ducts results in focal biliary cirrhosis. This presents by the age of fifteen years and is only seen in approximately 5% of individuals (O'Sullivan and Freedman, 2009).

In adults, men are almost always infertile. This is the result of congenital bilateral absence of the vas deferens. This is also the case for men who have only minor mutations of the CFTR gene and are otherwise asymptomatic. The vas deferens appears to be very sensitive to changes in the CFTR gene.

The most common symptoms of CF, however, relate to the pulmonary system.

Chapter 2

2.0 Microbiology of cystic fibrosis

Prior to antibiotic therapy and modern medicine, it was common for CF patients to die in infancy – most commonly from *Staphylococcal* infection (Govan & Deretic, 1996). CF patients in the modern era, however, remain susceptible to infection and colonization by a number of organisms, the most common being *Pseudomonas aeruginosa* – originally called *Bacillus pyocyaneus* (Govan & Deretic, 1996) – which affects over 90% of patients at some point during their lives and is responsible for the vast proportion of mortality and morbidity in CF patients (Lyczak *et al*, 2002). Other frequently isolated organisms include *Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex (Bcc) – the latter of which can lead to death by "Cepacia syndrome" (Isles *et al*, 1984; Aaron *et al*, 2000). Bcc has been responsible for many outbreaks in CF units around the world (Govan *et al*, 1993; Speert *et al*, 2002) and has been noted to increase the risk of death within one year of lung transplant by up to six times (Alexander *et al*, 2008). It is notable, however, that the actual strain causing this complex has yet to be identified with any rigor although *Burkholderia cenocepacia* appears to be predominant. To date, studies that have been carried out have only had very small numbers (LiPuma, 2005). Whilst colonisation by pathogens such as *Pseudomonas aeruginosa* and Bcc often represent a negative development in the clinical progress of a patient, it has also been found that the antifungal properties of these organisms may, in fact, inhibit further colonisation by *Candida* and other fungal species (Govan & Deretic, 1996).

Other organisms that have been found in the respiratory tract of CF patients include *Haemophilus influenzae* and *Staphylococcus aureus* (Geller, 2009) though, whilst often found, it is thought that both *S. aureus* and *H. influenzae* are not necessarily pathogens in CF patients (Lyczak *et al*, 2002).

Bacterial infection of the CF lung is almost entirely limited to the intraluminal mucus (Worlitzsch *et al*, 2002). Mucinases produced by the bacteria allow them to set up small protected niche environments in the lung. It has been found that they set up a complex micro-environment where oxygen diffusion is slow – creating anaerobic plaque formation. In addition, the easy biofilm formation may be enhanced by quorum factors and the lack of free motility (Belkin *et al*, 2006). In the low ASL environment, the lack of water and considerably enhanced mesh structure of static mucus may result in a selective pressure that allows environmental organisms to thrive. This may explain why soil organisms that are resistant to drought such as *Burkholderia cepacia* are able to predominate (Rogers *et al*, 2004; Rogers *et al*, 2005).

2.1 *Burkholderia cepacia* complex

Burkholderia species have been the cause of much stigma within the CF community. Patients live in fear of contracting these organisms, and often become social pariahs within their own community once colonised. Segregation and isolation of patients with these bacteria further enhance this problem which continues to blight the CF community and individual patients (Govan & Deretic, 1996). It was noted in 1995 in the USA that the median survival for patients without Bcc species colonisation was thirty years. For those patients that were, unfortunately, colonised with these organisms, the median survival rate dropped to just twenty one years (Aaron *et al*, 2000) Whilst there is still disagreement about the validity of such a strategy, colonised patients are often cohorted and separated from other CF patients. This can result in some disruption in their clinical management, but also further stigmatization (Govan & Deretic, 1996).

The *Burkholderia* species were first described as phytopathogens that cause soft rot of onion bulbs by Burkholder (Burkholder, 1950); the *Burkholderia cepacia* complex represents a group of very similar species that are often isolated from CF patients. The first described

cases where *Burkholderia cepacia* was isolated were in the late 1970s. It is relatively unusual to isolate these otherwise benign organisms from healthy individuals (LiPuma, 2005). Originally described as "genomovars", the specific differences found between each "genomovar" in further analysis prompted a new naming system whereby each "genomovar" was given a species specific name. There are currently few known species, such as *B. pseudomallei* (causing the potentially fatal melioidosis) although it is thought likely that others will emerge (Inglis *et al*, 2004). The number of members of the Bcc is constantly rising as novel species are identified – usually via RecA PCR; with *B. ubonensis* being one of the most recently proposed (LiPuma, 2005, Vanlaere *et al*, 2008). In addition to these, there are a number of novel species that belong to a complex within the Bcc called the K taxon group. These include *B. contaminans* sp nov and *B .lata* sp nov (Vanlaere *et al*, 2009). Examples of the Bcc group are given below:

B. cepacia, B. multivorans, B. cenocepacia, B. stabilis, B. vienamiensis, B. dolosa, B. ambifaria, B. anthina, B. pyrrocinia, B. pseudomallei, B. ubonensis sp nov, *B. latens* sp nov, *B. diffusa* sp nov, *B. arboris* sp nov, *B. seminalis* sp nov, *B .metallica* sp nov.

The identification of the Bcc is complicated further by the relative biochemical unreactivity. It is not often isolated in a routine laboratory, and is often misidentified. Examples of this occurring can be found in most historical collections of Bcc isolates. On further examination, it has been found that they can, with hindsight, be differentiated into the various species (LiPuma *et al*, 1990). The possession of β-lactamases has recently been a method by which investigators have been able to differentiate species of the Bcc. They demonstrated that whilst similar to each other, each chromosomally encoded β-lactamase differed in some way – and that they were species specific (PenA [*B. Multivorans*] to PenL [*B. thailandensis*]). From this, they were able to produce dendrograms and change the idenfification of a number of isolates from their collection (Poirel *et al*, 2009).

Isolation of Bcc species still remains tricky in the routine laboratory. It has often been mistakenly identified as *Pandorea*, *Ralstonia* or *Achromobacter* species. Another exacerbating problem is the relative rarity of isolation. The result is that most commercially available identification kits such as the API system for biochemical analysis are inadequate (LiPuma, 2005). The close genetic relatedness of the individual species also confound the issue further and make simple culture/biochemical techniques practically redundant.

2.1.1 *Burkholderia cepacia* complex in cystic fibrosis

A number of strains have been identified over time, as being particularly prevalent in CF and immunocompromised populations, and studies have found that the most frequently reported sites of isolation of Bcc has been the lower respiratory tract (approximately 31% of isolates), although other rare complications such as septicaemia (first reported in a 17 year old in 1980) do arise (Govan & Deretic, 1996). The epidemic (ET12) strain is dominant in Eastern Canada and the UK. This strain is unique to *B. cenocepacia* (Baldwin *et al*, 2008). PHDC an epidemic strain is seen in the mid-Atlantic region of the Eastern United States but has been seen in rare occasions in European patients – it was first detected in Italy in 2001 (McDowell *et al*, 2004; LiPuma, 2005). ET12 was thought to have arrived in the UK as a consequence of contact at a CF summer camp in Edinburgh in 1989. Both the ET12 and PHDC strains are *B. cenocepacia* and it is clear that this species may be more transmissible between patients than other Bcc strains (Baldwin *et al*, 2008). Studies have found that *B.cenocepacia* isolates found in patients often appear to be mutually exclusive from environmental strains and are often not genetically linked to environmentally isolated strains. Patient to patient spread, therefore, is clearly the main vehicle through which patients are colonised with this organism. *B. multivorans*, however, is regularly isolated in the environment and studies have demonstrated that colonisation of patients is likely to be through this route. The disparate distribution of isolates also suggests separate colonisation/infection events that affect each patient individually (LiPuma, 2005). Patient to

patient spread is much less common with this species (Baldwin *et al*, 2008). Other species (such as *B. dolosa* SLC6) have been found to infect multiple patients though this is a less common event (Biddick *et al*, 2003). Sporadic colonisation/infections have been found with other species such as *B. gladioli* though, again, this is uncommon and *B. gladioli* does not form part of the Bcc group. Interestingly, it has been found that those species that are most frequently isolated from the environment, are the ones least found in CF and other patients (LiPuma, 2005).

It appears that infection of CF patients generally occurs with a single strain – although transient co-infection has been documented (Bernhardt *et al*, 2003). Indeed, there is also evidence that in some patients, infection with Bcc species may be transient in nature. This is an area that needs to be studied further, however, as other, as yet unknown strains may be involved (LiPuma, 2005). It has also been suggested that colonisation of patients by Bcc species may go unnoticed for as long as two years. This appears to be anecdotal evidence, however, and may rely upon the quality of sputum sampling (LiPuma, 2005).

The so called "cepacia syndrome" described in 1984 (Aaron *et al*, 2000) is a condition featuring necrotizing pneumonia, fever, bacteraemia, leukocytosis and a raised erythrocyte sedimentation rate (ESR). It is thought that as many as 20% of colonised patients will succumb to this syndrome (Govan & Deretic, 1996). The syndrome has largely been attributed to *B. cenocepacia* – albeit without sufficient rigor to conclusively rule out other species/strains. It appears, however, that the ET12 strain predominates. It has yet to be established whether other Bcc species have a role to play in this syndrome, and if not, what significance their role is in morbidity/mortality. It has been established that other species have caused such outcomes – particularly *B. multivorans* and *B. dolosa* but it is clear that more work is required in this area – with larger numbers of strains/patients to assess patient outcome (LiPuma, 2005). It has to be noted, with respect to the above, that in most patients that are colonised by Bcc, the outcome will be very similar to those who have been

colonised by *Pseudomonas* species (Govan & Deretic, 1996). Historically, the most commonly found CF related Bcc isolates were of the *B. cenocepacia* group (formerly known as Genomovar III), and it is these that exclusively carry the ET12 transmissibility marker (McDowell *et al*, 2004).

2.1.2 Epidemiology of Bcc in cystic fibrosis

The reason for the rise of Bcc prevalence in CF populations has so far been difficult to explain with any certainty. The increased prevalence first became apparent in the 1980s and was thought to be largely as a result of selective pressure by aggressive antimicrobial chemotherapy. Nebulised colistin has been suggested as one of the prime suspects in this mechanism. There has been little scientific evidence to back up these assumptions, however, and the social and economic lifestyles of an increasingly adult population of CF sufferers have yet to be factored into the equation with a full explanation remaining somewhat difficult to offer.

The transmission of Bcc between patients, and in a nosocomial environment, has been difficult to prove. Only with the advent of molecular methods such as pulsed field gel electrophoresis (PFGE) has this become possible. It does appear that in most cases, chronically colonised patients will only harbour one strain throughout, though multiple morphotypes may appear (LiPuma *et al*, 1991). In more recent years, occasional "swaps" of strains have been noted – for example a patient colonised with *B. multivorans*, may acquire a more virulent strain of *B. cenocepacia* (Saiman & Siegel, 2004).

A number of case control studies have been carried out that suggest patient to patient transmission is a factor – particularly in the hospital environment. A benchmark study by LiPuma *et al* (1990) was carried out at an education camp. This study clearly demonstrated, using restriction fragment length polymorphism (RFLP) typing and contact tracing, that person to person spread was the primary method of acquisition (in this case). It did not

indicate the actual method of transmission – whether by natural means or via a fomite pathway, though it was one of the first and most reliable studies that proved the, so far, circumstantial observations (Govan & Deretic, 1996).

Whilst person to person contact has been demonstrated, Bcc has often proved difficult to isolate from environmental sources where CF patients have not previously been present. It has been demonstrated, however, that Bcc can and does contaminate the environment around a colonised patient. It seems highly likely, and a number of studies have confirmed this, that patient to environment transmission occurs. This includes sources such as respiratory equipment (such as nebulisers), disinfectants and furniture – such as taps and sinks (Govan and Deretic, 1996). Interestingly, it was found that transmission was often strain dependant. Patients who were colonised by an epidemic strain (such as ET12), remained chronically colonised. Where a patient possessed two strains, only the epidemic strain was passed to the close contact (Govan *et al*, 1993).

Strain type, whilst associated with colonisation status did not appear to affect clinical outcome. It was found in a study by Brown *et al* (1993) that whilst 50% of patients colonised by an epidemic strain eventually succumbed, a significant number of patients were stable for the period of the study. Evidence was not found that correlated colonisation with poor pulmonary status. However, it was found that poor pulmonary status was one of the risk factors for poor survivability once colonised (Brown *et al*, 1993; Govan and Deretic, 1996).

In recent years, the spread of colonisation appears to have moved from *B. cenocepacia*, to a greater incidence of *B. multivorans*. The detection of unique strains – largely assumed to be from environmental sources has now become commonplace in many centres (Baldwin *et al*, 2008, France *et al*, 2008)

2.1.3 Treatment and resistance

Whilst it is not possible to completely eradicate Bcc from the lungs – particularly the lower respiratory tract, it has been demonstrated in a number of studies that the effect of antimicrobial chemotherapy has a beneficial effect in terms of reduction of bacterial density. This, in turn, reduces the host immune response and can lead to both a qualitative and quantitative reduction in bacterial virulence and virulence factor production (Aaron *et al*, 2000). It has also been demonstrated that the net benefit from antimicrobials only comes with *combination* therapy – opening the way for synergy investigations of the useful antibiotic groups (Aaron *et al*, 2000).

Bcc species are very resistant to a broad spectrum of antibiotics including aminoglycosides and practically all of the available β-lactam antibiotics, and development of novel treatment therapies has been slow to evolve (Ciofu, 1996). Indeed, resistance has been demonstrated even in patients who are not currently undergoing antimicrobial chemotherapy. It has been found that even when susceptibility *in-vitro* appears to be promising, aggressive antimicrobial therapy often fails to yield good clinical improvement of the patient or quantitatively reduce the numbers of bacteria in cultures (Govan & Deretic, 1996). It is thought that the environment of the CF lung may, in fact, induce resistance such as active efflux pump mechanisms. The Bcc are closely related to *Pseudomonas aeruginosa*, and as such demonstrate some homology in their resistance such as the multi drug efflux mechanisms (Zhang *et al*, 2001). Numerous mechanisms of resistance have been noted/suggested, though a number of them are speculative. One of the mechanisms confers resistance to chloramphenicol, quinolones and trimethoprim (Aaron *et al*, 2000). Meropenem has been shown to be effective against Bcc species, but resistance upon multiple treatments does develop (Ciofu, 1996). Addition of a β-lactamase inhibitor with

carbapenems has been suggested but as yet, this has not been adopted. One of the most interesting aspects of the Bcc resistance spectrum is that of the highly inducible chromosomal *penA* gene that encodes for a β-lactamase type enzyme. This allows the bacterium to use penicillin antimicrobials as a substrate (Joris *et al*, 1993; Aaron et al, 2000). The alteration of penicillin binding proteins (PBPs) may also play a role (Aaron *et al*, 2000).

It has been found that the Bcc outer membrane features far less permeability than many other organisms (up to ten times less permeable than *E. coli*). It would appear that this has a wide ranging and broad spectrum effect not only on a wide range of antimicrobial agents, but also some disinfectants (Govan & Deretic, 1996).

Quorum sensing may also play a role, where gene expression is dependent upon bacterial density. Biofilm production and the actual cell wall structure also plays a role (LiPuma, 2005), and there is some burgeoning research into this area. It is thought that current susceptibility studies may not adequately predict the environment in which Bcc species (and, in fact other CF colonising organisms) exist.

It is the multiple resistance of Bcc species that make laboratory susceptibilities difficult to interpret. It is often the case that all or many of the single antibiotics tested are resistant *in-vitro* and thus combination testing is becoming a popular, though still debated method of predicting clinical success (Aaron *et al*, 2000). Additionally, where MICs are tested *in-vitro* and found to be sensitive, it has been demonstrated that clinical failure of an antimicrobial may still occur. This brings the relevance of *in-vitro* testing into some question. However, as stated previously, the *in-vitro* studies for this organism may still be relevant where empirical treatment is to be used (Ciofu *et al*, 1996; Petersen *et al*, 2006).

2.2 Stenotrophomonas maltophilia

Often seen as an environmental organism, *Stenotrophomonas maltophilia* is an increasingly important nosocomial pathogen and is resistant to many of the commonly used antimicrobial agents (Gabriel *et al*, 2004). It is found in a wide variety of environments and has been isolated from both soil and water habitats as well as animals. It has been found in diverse areas such as Japanese oil fields and Antarctica (Denton & Kerr, 1998; Valdezate, 2004). It has been found in a wide range of animals, both in faeces and in oral sites and has also been implicated in some animal diseases such as fleece rot in sheep (Denton & Kerr, 1998). It has also been suggested as a potential biological control since it is known to produce maltophilin – an antifungal agent that inhibits *Candida* and *Aspergillus* spp (Jakobi *et al*, 1996). Most of the species found in the genus *Stenotrophomonas* are environmental in nature and have been found in soil and agricultural settings (Yi *et al*, 2010). These include *S. ginsengisoli* (Ginseng fields), *S. koreensis* (compost), *S. dokdonensis* (soil) and *S. panacihumi* (Ginseng field). A study in 1999 by Berg and colleagues found that *S.maltophilia* isolates from environmental and clinical sources differed very little, with no clustering – suggesting that these two sources may not be mutually exclusive. In the process, it was noted that PFGE was the most discriminatory test.

Previously called *Xanthomonas maltophilia* and *Pseudomonas maltophilia*, *Stenotrophomonas maltophilia* is one of a small number of known organisms in its genus: *Stenotrophomonas*. The genus was proposed by Palleroni & Bradbury (1993) and the type strain was first isolated from an oro-pharangeal swab of a patient with oral carcinoma (Denton & Kerr, 1998). *S. maltophilia* is a motile, Gram negative curving or straight rod shaped bacterium in the order of 0.5–1.5 µM long. It is an obligate aerobe and grows

optimally at around 35^{o}C in an aerobic environment. Its colonial appearance on culture media is fairly typical of such "*Pseudomonas* like" organisms – flat, smooth and wet – often with a brown discoloured appearance (particularly in media with a high tyrosine content (Blazevic, 1976)). Whilst considered to be generally unreactive biochemically, *S.maltophilia* can be identified by means of the commercially available API20NE system (Biomerieux). *Stenotrophomonas maltophilia* is relatively easy to isolate and can be done so from a wide range of normally available media found in diagnostic laboratories. Common media such as Columbia blood, MacConkey and CLED agar will isolate this pathogen quite satisfactorily and the organism will grow overnight in temperatures ranging from 20-37^{o}C in air. *S. maltophilia* can also be isolated using blood culture systems, though the efficiency with which this is done is variable (Denton & Kerr, 1998).

Molecular methods to identify *S. maltophilia* are available; for example single strand conformation polymorphism electrophoresis of polymerase chain reaction (PCR) amplified 16s rRNA fragments. In addition to this, differences in internal transcribed spacers have been described that are species specific (Tyler *et al*, 1995; Denton & Kerr, 1998).

2.2.1 Antimicrobial testing

Whilst *S. maltophilia* are noted to be phenotypically stable when sequenced, an issue that is encountered in the laboratory is that of variability/heterogeneity. Indeed, this is a common factor with CF isolates. Aaron (2007) found that CF organisms frequently exist as multiple morphotypes. This reduces the accuracy and reproducibility of minimum inhibitory concentration (MIC), synergy and multiple combination bacterial testing (MCBT) methods and may render them useless or misleading to the clinician. In one study covering 101 sputum samples taken from patients, an average of four morphotypes of *P. aeruginosa* existed concurrently in each specimen with a mean of three different antibiograms from each morphotype. With this number of morphotypes, it was common to see as many as twelve

different susceptibilities when testing from one specimen. This is a commonly noted phenomenon with all of the species tested in this study. This variation in observable results is noted to be particularly prominent in the quinolones and often makes optimisation of antimicrobial therapy difficult (Denton & Kerr, 1998). Additionally, *S. maltophilia* is often isolated from mixed cultures, and this has stimulated some debate about its true role in CF pathology (Valdezate *et al*, 2004).

Broth/agar dilution methods have been recommended by the Clinical Laboratory Standards Institute (CLSI), although a study by Yao *et al* (1995), found 94% agreement between E-testing and the disc diffusion method. A number of reasons have been cited for the seemingly random variation in susceptibility testing including Zn^{2+} concentration of the test media (affecting Imipenem). Similar tests have been carried out to confirm these effects and have found that the same ion concentrations do not affect Meropenem. Equally both Ca^{2+} and Mg^{2+} ions do not appear to influence susceptibility testing (Hawkey *et al*, 1993; Denton & Kerr, 1998). It is also well documented that the incubation temperature can affect the appearance of both aminoglycosides and polymyxin B – appearing to be more resistant when tested at 30^{o}C. Current British Society for Antimicrobial Chemotherapy (BSAC) recommendations are that all isolates of *S. maltophilia* are tested at 30^{o}C to avoid false sensitivity reporting (Pappapetropolou *et al*, 1994; Wheat *et al*, 1985; Denton & Kerr, 1998). Another, more recent, variable that has been noted in the *S. maltophilia* species, is a novel small colony variant (SCV) that has resisted antimicrobial susceptibility testing and is difficult to grow consistently on standard media (Anderson *et al*, 2007). It is possible that this and potential other SCVs may induce further variation within any testing schedule and that whilst doing this, they may not be detected by normal means. These SCVs have been tested under non-standard conditions (chocolate agar with 48 h growth) with the result that they were found to have potential resistance to sulfamethoxazole-trimethoprim (unlike other *S. maltophilia* isolates). However, they were found to have some potential susceptibility to

minocycline and levofloxacin (individually) – indicating the possibility of tigecycline sensitivity.

2.2.2 Resistance mechanisms

S. maltophilia demonstrates resistance to broad spectrum antibiotics – particularly the β-lactams. Whilst it was thought that two enzymes were responsible for this, further enzymes have been identified – mainly in the metallo β-lactamase or serine class. Of the two enzymes most studied, L1, a metallo β-lactamase differs from that of other species types and is found in virtually all wild type strains (Denton & Kerr, 1998). Whilst this enzyme is dependent upon the zinc concentration, it can also be effective (though less so) in the presence of cobalt, cadmium or nickel ions. It has been shown to hydrolyse penicillin antimicrobials but has no hydrolysing effect upon aztreonam. It is also responsible for the effect commonly seen with the carbapenems (Denton & Kerr, 1998). This enzyme is not affected by β-lactamase inhibitors such as clavulanic acid, though other compounds have been shown to be effective in laboratory trials (Payne *et al*, 1997). L2 – the other enzyme that is found in *S.maltophilia*, is a TEM β-lactamase and in the serine class. It is responsible for the hydrolysis of aztreonam and also demonstrates activity against cephalosporins. It is inhibited by β-lactamase inhibitors such as clavulanic acid although is less susceptible to tazobactam or sulbactam inhibitors. Oddities have been reported in the regulation of expression of these enzymes – where strains are able to produce large quantities without induction, but these appear to be isolated cases of mutation (Payne *et al*, 1997).

In addition to enzymatic resistance mechanisms, it is thought that the poor penetration of penicillins and cephalosporins may enhance resistance. It is not known how the number or state of porin channels affects this (Denton & Kerr, 1998).

It is thought that aminoglycoside resistance is partly temperature related and also related to the O side chain of the lipopolysaccharides. The lower growth temperature and subsequent

change in the phosphate content correlating with enhanced resistance (Denton & Kerr, 1998).

Cross resistance with quinolones and doxycycline has been reported and this is thought to be related to changes in the outer membrane proteins (Denton & Kerr, 1998; Giacometti *et al*, 2000). In addition to doxycycline, active transport (energy dependent efflux) resistance mechanisms to tetracyclines have also been reported. This system is not active against aminoglycosides or β-lactam antimicrobials, but is effective against chloramphenicol and tetracyclines (Alonso & Martinez, 1997). As with *Pseudomonas aeruginosa* and the Bcc species, efflux pumps have been described that confer multiple resistance. Most of these are homologues of those described in *Pseudomonas aeruginosa* (Zhang *et al*, 2001).

2.2.3 Treatment options

As with other CF pathogens, the treatment of *S. maltophilia* infection is difficult. Past evidence suggested that cotrimoxazole (trimethoprim-sulfamethoxazole) would have been the drug of choice, although resistance has been noted to rise and fall with increased and decreased use of this drug respectively (Denton & Kerr, 1998). In light of the above evidence, it would be sensible to reject penicillins and most of the cephalosporins for empirical treatment. Ceftazidime, however, has been found to be active against *S.maltophilia* although this is highly variable and not, therefore, reliable for empirical treatment (Denton & Kerr, 1998).

A number of combination antibiotics have been tested against *S. maltophilia*, including ticarcillin clavulanate which demonstrated good activity. Other combinations, however, did not yield good results. These included amoxicillin clavulanate, ampicillin sulbactam and piperacillin-tazobactam (Denton & Kerr, 1998). Likewise, aztreonam and imipenem, in combination with β-lactamase inhibitors fared poorly. Where increased activity was noted,

it was found that plasma levels of clavulanic acid decrease sufficiently *in-vivo* to bring into doubt the usefulness of these combinations (Denton & Kerr, 1998).

Minocycline and doxycycline have been demonstrated to exhibit good activity against *S.maltophilia* (Gabriel *et al*, 2004) – contrasting with that of the legacy compound, tetracycline (Tilton *et al*, 1978).

2.3 Achromobacter xylosoxidans

Previously called *Alcaligenes xylosoxidans*, this organism is an unusual isolate from clinical material (Gabriel *et al*, 2004). *Achromobacter xylosoxidans* is an aerobic Gram-negative, non-fermentative rod shaped bacillus (Siebor, 2006). Oxidase positive and easily confused with *Pseudomonas* species, this organism was named by Yabuuchi & Ohyama (1971). Before being included in a single species type, this organism was recognised as a number of different biotypes – including: *Alcaligenes faecalis* (Moore & Picket (1960), and *Alcaligenes denitrificans* by De Ley *et al* (1970). Upon further testing it became apparent that all of these biotypes were actually the same species (Igra-siegman, 1980).

A. xylosoxidans is relatively easy to isolate – growing well on Columbia blood agar and MacConkey at 37^{o}C overnight. It produces glistening, smooth pinpoint colonies. *A.xylosoxidans* is able to be tested using the standard methods of other Gram negative bacteria. Where confusion exists as to the identification of this organism, the peritrichous flagella type may be identified, thus distinguishing *A.xylosoxidans* from *Pseudomonas* species (Igra-siegman *et al*, 1980). *A. xylosoxidans* can be identified in the routine laboratory, however, by the use of the API20NE identification system – and this has been found to be sufficiently accurate for routine use (Saiman *et al*, 2001).

2.3.1 Treatment options

As with other organisms found in CF, breakpoints are not routinely published by either BSAC, or CLSI and these can only, therefore, be extrapolated from other similar organisms or research publications.

A. xylosoxidans has been found to be resistant to most commonly used aminoglycosides and penicillins such as ampicillin and it has been suggested that its emergence as a CF pathogen may, along with *S. maltophilia*, be as a result of aggressive selection pressure caused by

antimicrobial chemotherapies (Cystic Fibrosis Medicine, 2008). Some sensitivity to chloramphenicol, tetracycline and colistin has also been described. Cotrimoxazole and Polymyxin B show good activity against *A. xylosoxidans*. A study carried out by Saiman *et al* (2001) found that *A. xylosoxidans* isolates were mostly resistant (97%) to conventional concentrations of tobramycin and colistin, though at the concentrations used in aerosol treatment, they were more susceptible (56% susceptibility). Upon testing, the organisms averaged 50% susceptibility to minocycline, imipenem, meropenem and piperacillin/tazobactam. Other agents (ciprofloxacin, tobramycin, cotrimoxazole, and chlorampenicol) did not fare well, with most of the isolates being resistant at normal therapeutic concentrations. Significantly, for this study, minocycline was found to be active against 51% of isolates tested in a study by Saiman *et al* (2001). None of the isolates tested, however, were CF related and this may have some bearing upon resistance mechanisms present.

2.3.2 Clinical relevance

The clinical relevance of *A. xylosoxidans* is, as yet, not fully understood (Saiman *et al*, 2001). Reports appear to suggest that colonisation by this organism does not necessarily lead to a decline in respiratory function (De Baets *et al*, 2007), though the presence of serum specific precipitating antibodies does appear to be linked with a decline in respiratory function (Ronne Hansen *et al*, 2006). Most authors, however, agree that increased antibiotic intervention is necessary (Ronne Hansen *et al*, 2006; De Baets *et al*, 2007).

2.4 Sequence of infection

The assumption is that CF patients are born without any lung colonisation. It is not uncommon, however, for over 30% of patients to have *Pseudomonas aeruginosa* isolated by their first birthday (Geller, 2009). A study carried out in the United States found that of 20,000 patients 29.8% of 2-5 year olds are colonised by *Pseudomonas*. In the 26-30 year

old group, the percentage rises dramatically to 81.3% (Doring *et al*, 2000). In the normal course of events, *Pseudomonas* will be isolated frequently – though not on every single culture, as transient colonisation develops. These strains are most likely to be planktonic but non-mucoid in their nature. The hypersensitivity at colonisation results in an acute lung infection that may be treated at this stage – with the possible eradication of *Pseudomonas* (Doring *et al*, 2000).

If left untreated, however, this colonisation later develops with the appearance of mucoid strains that embed themselves into an exo-polysaccharide matrix – a biofilm (Geller, 2009). As this biofilm builds, a type III hypersensitivity develops and antibodies are produced against numerous but specific antigens. The formation of immune complexes and the recruitment of neutrophils that decay appear to form areas of pus around the biofilms. It is thought that this may contribute to the obstruction of the airway in CF patients (Doring *et al*, 2000). Other bacteria are thought to be able to produce a similar effect in CF patients – even in the absence of *Pseudomonas*. A decrease in forced expiratory volume (over 1 s) – FEV1 has been demonstrated with other bacteria (Sharma *et al*, 1995). With this in mind, however, it is a fact that many patients are able to continue for years without a significant decrease of FEV1 – even in the presence of *Pseudomonas* or other bacterial colonisation (Doring *et al*, 2000). In most patients, however, mucoid *Pseudomonas* will result in a serious decline in FEV1. As stated previously, it is thought that this decline is primarily due to the increase in sputum production/inflammation and the increased release of serine proteinases. Other unrelated causes of exacerbations, however, cannot be ruled out (Doring *et al*, 2000).

2.5 Infection control

Modified and stringent infection control practices have played an enormous part in the reduction of infection and colonisation. The legislation and recommendations for infection control are legion and thorough (Saiman & Siegel, 2004). Infection control practices follow similar lines to those of other hospital areas – hand hygiene, segregation and the observation of equipment precautions – such as the cleaning of nebuliser equipment. The organisms isolated in CF patients are environmental in nature, and survive for prolonged periods on hard surfaces in the hospital and home environment.

A number of studies have found that isolates in CF, which are effectively environmental organisms (Rogers *et al*, 2004; Valdezate *et al*, 2004; Rogers *et al*, 2005), are often unrelated and may originate from multiple, independent sites/sources (Denton *et al*, 1998; Denton & Kerr, 2002; Valdezate *et al*, 2004) – this is particularly true of *S. maltophilia* and *B. multivorans* isolates. Isolation of patients, it has been suggested, was not necessarily indicated as based upon the findings of these studies, it would appear that one of the most important factors in acquisition of colonising organisms is the environment from which they originate. There are many potential sources of infection/colonisation with *S. maltophilia* and other CF isolates. One example where the spread of organisms was studied found that neubulizer use of antimicrobials such as tobramycin may have assisted the acquisition of *S.maltophilia* with 16% of patients receiving nebulised tobramycin and 22% receiving nebulised placebo acquiring new strains (Denton & Kerr, 2002). However, whilst the segregation of patients to protect against acquisition of *S. maltophilia* is not necessarily indicated, the situation is very different for patients that are colonised with Bcc (Speert *et al*, 2002). Another study investigated the use of nebulisers but found mixed results. Those patients who practiced good nebuliser hygiene and followed the recommended instructions for use were found to have minimal or no contamination from nebulisers (Hutchinson *et al*,

1996). Interestingly, the number of contaminated nebulisers was noted to be high, but the transmission rates were relatively low – for example 7 out of 35 patients using a nebuliser were positive for either Bcc species or *S. maltophilia*. The conclusions of the study were that most transmission of CF related organisms, must, therefore originate from other environmental sources. This is further backed up by a more recent study by Valdezate *et al* (2004) where a large number of *S. maltophilia* isolates were investigated by the use of PFGE. A high genetic diversity was found, despite all of the isolates being isolated at the same hospital.

The acquisition of Bcc is for most patients, a great concern. Aside from the obvious physical health implications, there are other concerns. The psycho-social implications of infection control regulations within CF units and at other social gatherings where CF patients are likely to come into contact can be considerable.

Burkholderia cepacia complex organisms are noted to be easily transmissible between patients on a unit (Speert *et al*, 2002) and so one may expect that isolates found in the same unit would be of similar relatedness – particularly strains of *B. cenocepacia*. The study by Speert *et al* (2002) however, also demonstrated that it remains difficult to accurately predict the transmissibility of these organisms. Their study found that *B. cenocepacia* (formerly Genomovar III) was the most transmissible strain type, and that the ET12 epidemic strain was the most "robust" marker of transmissibility. *B. multivorans* in most studies have not been isolated in the same numbers, leading to many authors stating that it is a relatively uncommon CF pathogen when related to *B. cenocepacia* (Saiman & Siegel, 2004), though in recent years, there has been a reduction in the number of *B. cenocepacia* isolates – presumably as part of a concerted and rigorous infection control policy. It has been demonstrated that *B. multivorans* is primarily contracted from environmental sources, unlike *B. cenocepacia* where patient-patient spread is the most common vehicle. It is, therefore, more difficult to prevent the colonisation of patients by *B. multivorans* than *B. cenocepacia*.

Despite this change in relative distribution/prevalence, there have been noted cases where patients colonised with *B. multivorans* or other Bcc species, have "swapped" isolates. This change of colonisation status from *B. multivorans* to the much more transmissible *B.cenocepacia* has resulted in a severe and rapid decline in respiratory function with catastrophic results for the patient (Saiman & Siegel, 2004).

Recommendations have been made, that suggest it may be possible, and even prudent to cohort patients based upon transmissibility markers that are detected in their particular isolates of Bcc species. However, it has been noted that some of these markers such as *cblA* and BCESM have been noted to reside upon unstable choromosomal regions – and this may render such practices unsafe (Mahenthiralingam *et al*, 1997; McDowell *et al*, 2004).

Other organisms that have been noted in CF patients, present differing infection control risks. The emergence of *S. maltophilia* and *A. xylosoxidans* (the latter being less common with prevalence rates reported around 10%) has increased the complexity of infection control procedures and antimicrobial chemotherapy, in that the routes of transmission, and indeed the clinical relevance, of these organisms may sometimes be in some doubt (Cystic Fibrosis Medicine, 2008).

The Leeds Teaching Hospitals NHS Trust (LTHT) CF unit has followed the recommendations given by the CF Trust, and outlined in their document: "The *Burkholderia cepacia* complex – suggestions for prevention and infection control" (2004). As stated earlier, the consequences of separation may be psychologically undesirable to patients, but it is considered necessary and as can be seen from the data presented in this, and past studies, it has been successful (CF Trust Guidelines, 2004). Since the Bcc species (in addition to the more common *Pseudomonas aeruginosa*) are the most transmissible and potentially harmful strains, so it follows that infection control practices must seek to eliminate these organisms above all other social concerns.

Stringent infection control measures such as those applied in the LTHT have been demonstrated to significantly reduce the incidence of patient-patient spread of Bcc and other pathogens. Likewise, these studies have also demonstrated that the emergence of environmental strains cannot easily be stemmed by such measures, as they remain largely outside the control of the hospital and clinician (Baldwin *et al*, 2008). One result of good practice within CF units has been the reduction in *B. cenocepacia* colonisation rates. Conversely, and seemingly in line with our historical collection, the prevalence of *B.multivorans* has risen in the last decade – indicating again, that environmental factors may influence the rates of colonisation (Baldwin *et al*, 2008) – this was noted in Vancouver during a study carried out by Mahenthiralingam *et al* (1997) where little cross infection by *B.multivorans* was detected from a 17 year period. It is thought that this rise in prevalence may be largely due to the fall in colonisation with *B. cenocepacia* isolates, rather than a direct rise in the number of *B. multivorans*. The isolates that were collected by the microbiology department of LTHT would go some way to supporting this trend, with more distinct *B. multivorans* isolates being recorded over the 10 year period, than the more transmissible *B. cenocepacia* (Biddick *et al*, 2003). It is clear that the distribution of species within the CF community is changing, and this may result from aggressive antimicrobial pressures, but is most likely to be due to thorough infection control practices (Reik *et al*, 2005).

Chapter 3

3.0 Antimicrobial chemotherapy

The benefit of early prophylactic antibiotics has been questioned in the past – with some data suggesting that increased antibiotic use can be directly correlated with the rise in *Pseudomonas* colonisation and infection. It is though that this may arise as a result of the suppression of the normal respiratory flora – thus paving the way for increased colonisation by *Pseudomonas* due to the lack of competition for resources. It has been demonstrated during *in-vitro* studies that combined therapies (such as oral ciprofloxacin with nebulised colistin), can potentially reduce the numbers of bacteria in the CF lung to almost undetectable limits though it would appear that the *in-vivo* situation may differ significantly. It is thought, however, that early therapy before colonisation may delay the onset of *Pseudomonas* infection (Valerius, 1991). Intensive treatment has been demonstrated in some studies to reduce the onset of chronic *Pseudomonas* colonisation in over 80% of cases (Frederiksen *et al*, 1999). Studies similar to this have been carried out using a range of different anti-*Pseudomonal* drugs such as ceftazidime and tobramycin. It seems at present, however, that no rigorous (prospective) studies have been carried out where antibiotics are given to prevent *Pseudomonas* colonisation before colonisation is noted (Doring *et al*, 2000).

Another problem in treatment of CF patients is the mucoid *Pseudomonas* strain. It has been proved virtually impossible to eradicate these strains when they colonise patients. This is because it is difficult to achieve therapeutic concentrations of antibiotics that can penetrate the biofilm layer, and kill the organisms. This may also be a problem with other colonising organisms such as those tested in this study. In addition to the polysaccharide matrix in the

biofilm, the low ASL volume along with mucus plugging may also act to restrict antibiotic access (Doring *et al*, 2000).

Antibiotics are thought to penetrate the bronchial secretions via the blood. This is a process of passive diffusion and differs with each antibiotic type (Doring *et al*, 2000). Inhalation of antibiotics is a common form of treatment with CF patients – with tobramycin and/or colistin being the most commonly used. This ensures high therapeutic doses at the site of infection. Drugs given parenterally will necessarily require much higher doses – raising issues of toxicity and practicality where the patient is self administering at home (Doring *et al*, 2000).

Much has been said of *Pseudomonas* in CF, but there are other pathogens associated with pulmonary infection and colonisation and the treatment of these bring a number of difficulties, such as pan-resistance and antibiotic access to biofilm layers. These organisms include the *Burkholderia cepacia* complex and its associated species as well as *S.maltophilia* and *A. xylosoxidans*.

3.1 Amikacin and tobramycin

Both of these antibiotics are aminoglycosides – bactericidal protein synthesis inhibitors. Derived from *Actinomycetes*, their polycationic aminoglycoside structures bind to the outer membrane of the bacterial cell. They will also bind to the anionic phospholipids of mammalian cell membranes. Aminoglycosides also bind to the proximal tubular cells – bringing about some of the toxicity of this class of antibiotic. Transport across mammalian cell membranes is generally restricted because of the hydrophobicity of these antibiotics.

Due to the cationic nature of these antibiotics, they bind to the negatively charged sites on the outer bacterial membrane. This binding is sufficient to disrupt the membrane integrity and it is this process that may be one of the two main modes of action of this antibiotic

group. These antibiotics also bind to the 30S ribosomal subunit causing an alteration in the codon-anticodon recognition mechanism. This results in misreading of mRNA and the subsequent production of nonsense proteins. They have also been found to bind to the 50s ribosomal subunit, although the effect of this is unclear (Craig & Stitzel, 2003). They enter the cell via an oxygen dependent active transport system (that may be blocked when the patient is treated with chloramphenicol). It is thought that there may be more than one mode of action, as protein synthesis inhibitors are usually not so rapid in their killing of the bacterial cells. One phenomenon that has been noted with aminoglycosides is their synergy when used in combination with β-lactam antibiotics. It is thought that this is because the administration of a β-lactam antibiotic will reverse the often seen effects of low oxygen tension and low pH. These effects are often noted in bacterial abscesses. If the aminoglycoside alone were to be administered, then penetration into the cells would be inhibited as the active transport is dependent on the latter conditions being optimal. Prior administration of the β-lactam antibiotic will effectively prepare the ground for administration of the aminoglycoside – resulting in a synergistic effect (Craig and Stitzel, 2003).

Gentamicin is the most commonly used aminoglycoside – often in cases of sepsis. tobramycin, however, is the aminoglycoside of choice against *Pseudomonas aeruginosa*. tobramycin is widely used to treat CF patients.

Aminoglycosides are not well absorbed – being given intravenously or intramuscularly. They are not absorbed in the GI tract, exhibit minimal binding to plasma proteins, and do not cross the blood brain barrier. Accumulation can occur in renally impaired patients, as virtually the entire antibiotic is eliminated by glomerular filtration in the kidneys, resulting in renal toxicity. Likewise, in the tissues, it is possible to reach toxic levels where the dose is continuous and not monitored carefully.

One advantage of the aminoglycosides is the long duration of their post antibiotic effect. Even after the levels of antibiotic fall below the sub-inhibitory level, re-growth of the bacterial pathogen is restricted. It is thought that this may be due to the time taken for the bacterial cells to repair/replace the ribosome. This means that aminoglycosides such as tobramycin can be given as a once daily dose – making administration much simpler for CF patients.

3.1.1 Resistance mechanisms

One of the main resistance mechanisms in the aminoglycosides is the plasmid mediated production of enzymes that are capable of phosphorylating, adenylating or acetylating the antibiotic – usually by attacking the first ring structure. *Pseudomonads* resist the transport of aminoglycosides in to the cytosol and the presence of multiple active transport pumps in bacteria such as these cause significant issues.

3.2 Ceftazidime

Comprising a 7-aminocephalosporanic acid composed of a hydrothiazine ring fused to a β-lactam ring, this third generation cephalosporin has the greatest activity against *Pseudomonal* pathogens of all of the cephalosporins (Craig & Stitzel, 2003). Ceftazidime is a semi-synthetic antibiotic of the cephalosporin class. These drugs are derived from a number of microorganisms such as *Cephalosporium* and *Streptomyces*. They are grouped according the stability of their β-lactam ring structure; each group varying in their antimicrobial spectrum of activity. As with other β-lactam antibiotics, the mode of action is primarily aimed at the cell wall synthesis of the bacterium. β-lactam antibiotics closely resemble the terminal D-alanyl-D-analine part of the pentapeptides of the peptidoglycan (Craig & Stitzel, 2003). The covalent bonding of these transpeptidases results in an acyl-enzyme molecule that is stable and inactive. The resultant structure prevents further enzyme functions but requires the β-lactam ring structure to remain intact during this process. The

penicillin binding proteins (PBPs) are also involved in the assembly and maintenance of the peptidoglycan cell wall. There are a number of these that may be inactivated by the β-lactam antibiotic which results in a weaker cell wall that may deform the bacterium or cause lysis.

Ceftazidime is available either intramuscularly or intravenously – giving good bioavailability. It is one of the limited number of cephalosporins that reach therapeutic concentrations in cerebrospinal fluid. In addition, the third generation cephalosporins are among the most useful in the treatment of pulmonary infections (Craig & Stitzel, 2003).

Ceftazidime is excreted primarily via the kidneys and as such, the dosage may need to be adjusted in renally impaired patients.

3.2.1 Resistance mechanisms

Whilst β-lactamase enzymes can inactivate ceftazidime, they are much less efficient than the cephalosporinases – β-lactamases that are specific to this group of antibiotics. It is these β-lactamases that hydrolyse the β-lactam ring structure, rendering it useless. These enzymes are derived from the PBPs and bind to the β-lactam antibiotics. This then forms an acyl-enzyme molecule that can be deacylated and ultimately hydrolysed (Craig & Stitzel, 2003). The genes for these β-lactamase enzymes are often highly inducible (where they are chromosomal) and mobile – being transported via plasmids and transposons.

Pseudomonas and some other Gram negative organisms are able to actively pump out the antibiotic from the cell. The multi drug efflux mechanisms are often associated with multiple resistance and are a major problem in CF patients that are colonised with strains such as Bcc.

The other mechanism of interest with respect to Gram negative organisms is the reduction in access to PBPs. Clearly, if the antibiotic is unable to bind with these proteins, then it will be

rendered ineffective and clinical failure will result. Gram negative organisms, by virtue of their outer cell membrane, are able to reduce access to large molecules such as β-lactam antibiotics. This can be done by mutating the porin channels and is often seen in *Pseudomonas* species.

Ceftazidime, in common with other cephalospirins of the third generation may induce the production of ESBL enzymes in *Pseudomonas* and other environmental colonisers. These are highly transferable resistance mechanisms and can result in further cross resistance – particularly in a ward environment (Craig & Stitzel, 2003).

3.3 Piperacillin/tazobactam

Piperacillin along with ticarcillin and mezlocillin are anti-*Pseudomonal* penicillins. Piperacillin is generally combined with tazobactam which acts as a β-lactamase inhibitor. It is often used to treat CF related *Pseudomonas aeruginosa* and also pneumonias in patients that have been ventilated. It can only be given parenterally and is formulated as a sodium salt which may cause problems where older patients have congestive heart failure. As with the cephalosporins, piperacillin is primarily excreted through the renal system and thus would require some dosage adjustment in renally impaired patients. Piperacillin, as a β-lactam antibiotic is susceptible to many of the same mechanisms as other β-lactams.

3.4 Meropenem

A bacteriocidal β-lactam antibiotic from the carbapenem family, meropenem is similar to imipenem in its spectrum of action. Meropenem is a stable antibiotic and does not, therefore, have to be combined with cilastatin for administration, unlike imipenem. The carbapenems were developed as broad spectrum antibiotics that were capable of dealing with β-lactamase induced resistance. One of the most notable side effects of this class of antibiotics is the neurotoxicity that can be induced by high plasma concentrations, though meropenem is less likely to induce seizures than imipenem. Meropenem is active against

Pseudomonas and most *Enterobacteriaceae*, although resistant strains have emerged. It has also been shown to have good activity against *Burkholderia* spp although resistance, once again has been noted (Ciofu *et al*, 1996). Meropenem is clinically ineffective against organisms such as *Stenotrophomonas maltophilia*.

Whilst meropenem is generally well tolerated, there are a number of possible side effects, including nausea, dizziness and "drug fever". It may, however, be useful in cases where β-lactam drugs are not indicated due to hypersensitivity (Ciofu *et al*, 1996).

3.5 Aztreonam

This is a monobactam antibiotic – comprised of a monocyclic β-lactam ring and a substituent at the R-3 position. Active against *Pseudomonas*, *Neisseria* spp and *Haemophilus*, it is notable for being active only against aerobic Gram negative rod bacteria as it has only a low affinity for penicillin binding proteins. It is stable against β-lactamase enzymes – both chromosomal and plasmid mediated, though is susceptible to extended spectrum β-lactamases (ESBLs).

Aztreonam, as the only clinically available member of the monobactam group, is administered parenterally with a half life of 2 h and is broadly similar in its pharmacokinetics to comparable cephalosporins. It also has the added benefit of being viable in patients with type I hypersensitivity causing penicillin allergy. It is noted to be useful against *P. aeruginosa* (Shawar *et al*, 1999), though more variable where tested against Bcc species. Activity against *S. maltophilia* and *A. xylosoxidans* is noted to be relatively poor (Denton, 2008).

3.6 Ciprofloxacin

This is the most frequently used of all fluoroquinolone antimicrobial agents and is classed as a second generation antibiotic. It is active against the DNA gyrase topoisomerase II – an

enzyme that prevents supercoiling of DNA by strand cutting. An additional target unique to the fluoroquinolones is the topoisomerase IV DNA gyrase – this is responsible for the separation of the daughter cell after replication (Craig & Stitzel, 2003). Transcription and replication are inhibited by these actions, leading to a dual effect. The initial effect of ciprofloxacin is bacteriostatic. However, when the bacterial cell is unable to repair its DNA lesions, ciprofloxacin becomes bactericidal. It is active against both Gram positive bacteria and negative bacilli (GNB) and has been widely used against *Pseudomonas aeruginosa* in CF patients – its effectiveness due largely to the piperazine moiety against which resistance is increasing (Craig & Stitzel, 2003). It also has excellent activity against other Gram negative *enterobacteriaceae* and some environmental Gram negative organisms. Ciprofloxacin can be given orally, with a half life of approximately 3.3 h and has a limited number of side effects including GI tract upsets, hypersensitivity reactions (to theophylline in athsmatics) and, more rarely, CNS problems. It is well absorbed and often concentrates in both tissues and phagocytes with concentrations in extravascular spaces often exceeding those of the serum. Ciprofloxacin killing is concentration dependant, and as such it demonstrates a long post antibiotic effect (Craig & Stitzel, 2003).

Ciprofloxacin is absorbed rapidly after oral administration. It is excreted via the kidneys and may also be metabolized by hepatic conjugation and glucorinidation. Patients with hepatic or renal insufficiency, therefore, may need altered dosing (Craig & Stitzel, 2003).

Whilst the use of ciprofloxacin in pregnant women and children is not generally indicated, it is used in CF children that have been colonised by *Pseudomonas*.

3.6.1 Resistance mechanisms

One of the main resistance mutations occurs in the DNA gyrase A gene (gyrA). Lower levels of resistance are associated with topoisomerase IV with the primary mutation site being gyrA. Active efflux and the alteration of porin proteins also reduce the uptake of

Ciprofloxacin into the bacterial cell. It would appear that this resistance is chromosomally mediated.

Additionally, ciprofloxacin is noted to suffer from some variability when used against certain groups of organisms – primarily *S. maltophilia*. It is unclear whether this variability occurs clinically and affects patient outcomes, though *in-vitro* testing can be difficult to interpret, further adding to the problems created by the heterogeneity of *S. maltophilia* in antimicrobial testing (Denton & Kerr, 1998).

One of the main determinants of resistance to quinolones is the presence of multi drug efflux mechanisms. These have been described across CF colonising species and are generally homologous and highly conserved. Additionally, it has been suggested that some quinolones may actually provide a substrate for these mechanisms – particularly those that are active primarily against Gram positive species. There has been suggestion that pump inhibitors may be of use, though this is still largely unproven (Zhang *et al*, 2001).

3.7 Colistin

Produced from *Bacillus colistinus*, colistin is a polymyxin antibiotic (polymyxin E). It has a cationic property and interacts with the phospholipids of the cell membrane. This causes disruption to the structure, giving a bactericidal action with Gram negative organisms such as *Pseudomonas* and the *enterobacteriaceae*. It is not well absorbed from the GI tract and its use is limited by the severe neuro and nephro-toxicity reactions. It is generally used as a topical preparation in the eyes, ears and in skin infections where appropriate and is not generally indicated for other conditions where third/fourth generation cephalosporins are available. It may be used in clinical situations where treatment options are more limited – such as in CF patients. Colistin was largely overtaken by the use of aminoglycosides due to its high toxicity. It has since, however, become a widely used antimicrobial and has been demonstrated to be effective against organisms such as *Pseudomonas* in CF patients. There

are two available forms of colistin (colistin sulphate – mainly used topically, and sodium colistin methanesulphonate – used parenterally). Both forms of colistin are available for nebuliser administration, and as such are useful for home administration by CF patients. Colistin methanesulphonate is the less toxic variant and is, therefore, more widely used in nebuliser form as colistin sulphate has been noted to cause respiratory irritation. The exact mechanisms of toxicity of colistin are not fully understood, but nephro-toxicity may be an issue with some patients, though this can often be reversed upon suspension of treatment (Li *et al*, 2005).

Colistin kills very rapidly, by binding to the bacterial cell membrane. It displaces divalent cations from negatively charged phosphate groups of membrane lipids with the result that the cell membrane is catastrophically damaged.

3.7.1 Resistance to colistin

One benefit of the mechanism of action of this antibiotic is that it does not depend upon or affect any metabolic process within the bacterial cell. Since this is where most resistance mechanisms undergo rapid development, colistin has seen a slower rise in resistance rates than comparable aminoglycosides such as tobramycin (Li *et al*, 2005). Data relating to acquired resistance is limited though it is clear that this occurs where colistin is used over a period of time to treat *Pseudomonas* infections/colonisation. Some cross resistance has been noted between colistin and other polymyxin antibiotics. Unusually, it has been noted that where strains of *Pseudomonas* become more resistant to colistin, they may become more susceptible to a small number of other antibiotics – notably chloramphenicol and the tetracyclines. A number of multi-drug efflux systems have been noted – though these are not thought to be specifically related to colistin – resistance being almost a by product as colistin is not noted to penetrate the cell sufficiently. Additionally, a number of outer

membrane proteins that are able to change the valency of lipopolysaccharides in the cell memebrane have been noted to inactivate colistin and other polymyxins (Li *et al*, 2005).

3.7.2 Susceptibility testing

It has been noted that the killing effect of the two different preparations of colistin differs, with colistin sulphate being the most potent, but less used agent overall. There is some question over the *in-vitro* susceptibility where the sodium colistin methanesulphonate is to be used, as most susceptibility tests use the sulphate preparation.

3.8 Minocycline

The legacy derivative of tigecycline, minocycline is one of the tetracycline class of antibiotics. Tetracyclines act by inhibiting protein synthesis at the 30s ribosomal sub unit, and as such are bacteriostatic agents. They prevent the binding of aminoacyl transfer RNA to the receptor site. Tetracyclines are taken up into the bacterium by the process of active transport, causing an accumulation of the drug inside the cell. This is often the target of tetracycline resistance mechanisms. Tetracyclines are broad spectrum and are active against both Gram positive and Gram negative bacteria. Minocycline in particular is active against *Neisseria meningitidis* and is often used to eradicate nasal carriage of this organism.

Minocycline is useful orally or parenterally and will be found in most of the fluid compartments of the body as it is one of the most lipid soluble of the tetracycline class. Additionally, both minocycline and doxycycline are the only two tetracyclines that feature improved absorption when administered after food. Given the serum half life of Minocycline (16 h), it is considered to be the longest acting member of the tetracyclines. It is the good distribution qualities of minocycline that render it useful against mixed infections of the pulmonary system.

Minocycline is primarily excreted via the faeces, and reaches high concentrations in the bile – up to five times that of the serum concentration.

3.8.1 Resistance mechanisms

Most resistance to tetracyclines is conferred via plasmids. The development of energy dependent efflux mechanisms allow the bacteria to transport minocycline out of the cell – a mechanism which is sufficiently conserved to result in multi-resistance. It is common to find that resistance to one tetracycline class antibiotic will also be found with other classes – often with the exception of minocycline. It has been found that sub-inhibitory concentrations of the drug are able to induce resistance by the production of two specific proteins that block the uptake of tetracyclines through the cytoplasmic membrane.

3.9 Temocillin

Related to ticarcillin, temocillin is one of the β-lactamase stable antibiotics. It is non-toxic and usually well tolerated but its lack of activity against Gram positive organisms has resulted in it being "forgotten" as a useful antimicrobial (Livermore & Tulkens, 2009). It fails to induce β-lactamase production and should not, therefore, show antagonism when tested or used in combination with other antimicrobials. The problem resulting from this stability (temocillin is able to prevent the activation of serine preventing haemolysis), is that its binding to PBPs is dramatically impaired. The principal binding site for this antimicrobial is PBP 3 – and this may be one of the reasons for its lack of activity against Gram positive organisms (Livermore & Tulkens, 2009). Temocillin however, is considered to be one of the most active antimicrobials when compared with meropenem, imipenem, piperacillin and ceftazidime and has been shown to be of benefit in pulmonary exacerbations of CF (Lekkas *et al*, 2006). Cross-resistance is rarely seen with other β-lactam antibiotics, and temocillin can be administered twice daily – making it a convenient and useful antimicrobial (Lekkas *et al*, 2006). Temocillin has been used for a number of years and has

been shown in that time to be useful against both *Pseudomonas* and *Burkholderia* species (Taylor *et al*, 1992, Lekkas *et al*, 2006). Whilst *Pseudomonas* species do have a high MIC to temocillin – possibly related to their efficient efflux, reduced binding and poor penetration; *Burkholderia* spp have in some studies, also been shown to be more susceptible to temocillin than to other β-lactam antimicrobials. MIC90s of 32 mg/L have been reported (Livermore and Tulkens 2009). Temocillin is usually administered via a continuous infusion, and evidence suggests that high doses can be tolerated, and it is recommended as a specialist agent for patients with ventilator associated pneumonia, and where ESBL producing organisms are suspected.

Temocillin is resistant to many of the extended spectrum β-lactamases such as TEM, SHV, and CTX-M enzymes as well as AmpC (Livermore and Tulkens, 2009). It has been used in cases of colonisation of CF patients with *B. cepacia* and a clinical improvement was observed. Even in cases where temocillin was resistant *in-vitro*, a clinical improvement was noted – indicating that temocillin may still be useful at high MIC values (Taylor *et al*, 1992).

3.10 Tigecycline

Tigecycline (formerly GAR-936) is the 9-*t*-butylglycylamido derivative of minocycline, a new antimicrobial in the glycylcycline class and exhibits broad spectrum activity against extended β-lactamase producing organisms. It was first licenced in 2005 by the US Food and Drug Administration. Resistance to other tetracyclines usually occurs as a result of alterations to the tetracycline efflux pump or ribosomal protection. Tigecycline has been developed to overcome these mechanisms and does so by evasion of the Tet (A-E) efflux pumps. Tigecycline also attaches to the Tet (M) related ribosomes – possibly in a different orientation to previous tetracyclines – thus avoiding the resistance conferred by this mechanism, though it has been noted that tigecycline is likely to be susceptible to some multidrug efflux pumps such as those found in *Pseudomonas species* (Livermore, 2005). It

has been shown to be effective against both Gram negative and positive organisms, and does not show toxicity to patients with renal impairment (Kasbekar, 2006). Activity has been demonstrated against relevant isolates such as *Stenotrophomonas maltophilia* and *Acinetobacter spp*. Tigecycline is able to bypass many resistance mechanisms including the multidrug efflux pump mechanism (Pankey, 2005a, Pankey, 2005b). Studies have found, however, that this antibiotic agent shows only limited activity against *Proteus spp* and *Pseudomonas aeruginosa* (Milatovic, 2003). Tigecycline is considered to be a useful alternative for use against CTX-M producing *E.coli*. This is particularly useful as tigecycline is currently licenced for use in complex intra-abdominal infections as it shows excellent penetration into soft tissues.

Tigecycline works by binding to the 30S ribosomal sub unit – thus blocking the entry of tRNA. This mechanism, therefore, prevents protein synthesis by interrupting or halting the building of amino acids into peptide chains. The affinity of tigecycline to the ribosomal subunit is increased by up to five times by the addition of an N,N,-dimethylglyclamido group at the nine position of the minocycline molecule (Greer, 2006). It is not subject to efflux or ribosomal protection mechanisms and is active against organisms that, under other circumstances, would demonstrate tetracycline resistance determinants (Petersen *et al*, 2006).

3.10.1 Susceptibility testing

Tigecycline is notable in that it shows some variation when tested upon different media. MICs have been found to be one dilution higher when tested on Mueller Hinton agar instead of isosensitest agar, though how this translates to clinical practice is as yet unclear. Likewise, stored broths or long term stored media may render the drug more prone to oxidative damage though this is not noted to affect either disc testing or E-testing (Livermore, 2005).

Chapter 4

4.0 *In-vitro* synergy testing of organisms by E-test

The recommendation made by Peterson & Shanholtzer (1992) with regard to antimicrobial testing in the laboratory was that it should be reproducible, standardized and clinically relevant – this is still particularly relevant and presents numerous problems for testing in CF. They recommended the use of serial dilution methods that match as closely as possible the likely concentrations achievable *in-vivo*.

Fig 4.1 E-testing both alone (top left) and in combination (tob right and bottom) – note the combination elipse (see Chapter 5 for detailed method).

This was not recommended for routine testing but for on-demand testing where the clinical need was apparent. Additionally, it was suggested that these results should not be taken at face value, and that a clinical interpretation would have to be applied before patient

treatment commences. One of the interesting points made was the idea of reproducible and standardised serial dilution tests carried out in the laboratory. The E-testing method used in this study is the modern equivalent of this and can be seen in Fig 4.1 above.

The E-test – or "epsilometer" is an agar diffusion method that uses a concentration gradient of a selected antimicrobial substance. This is placed onto an agar plate inoculated with the target organism and is then incubated overnight. The result is an elliptical zone that intersects at the minimum inhibitory concentration of the gradient – the MIC (Fig 4.1). E-tests are available for most antimicrobials and are a quick and useful method of MIC testing for the non-specialist routine laboratory (White *et al*, 1996).

Synergy has, in the past, been determined by older established methods: checkerboard and time kill assays.

The checkerboard assay uses serial twofold dilutions of two antibiotics of different classes. These dilutions span the entire range of antimicrobial MICs from susceptible to resistant. The calculation used to determine synergy is identical to that used for E-testing. The time kill assay uses a similar method of broth dilutions but the concentrations differ slightly – reflecting ¼ to 2x the MIC for the test antibiotic when used with the particular bacterial species. These are then incubated overnight and sub-cultured at 0 h and 24 h respectively. The resulting colony count is used to define synergy (which is defined as $\geq$ 100 fold decrease in colony count at 24 h when compared to the original inoculum). Concordance of results has been demonstrated with the older methods and the E-tests and there are a number of studies where all three methods have been compared (Saiman, 2007). One example is a study by White *et al* (1996) where comparisons were made, and the E-test/checkerboard method were found to have between 75% and > 90% concordance, with similar results recorded between the E-test method and the time-kill assay (Bonapace *et al*, 2000; Orhan *et al*, 2005; Balke *et al*, 2006) though there have been occasional instances where this has not occurred. Further studies have been carried out with specific organisms – of note, a large

study of *S. maltophilia* where 94% concordance with the traditional broth microdilution method was found over a range of 176 isolates and sixteen antibiotics (Yao *et al*, 1995).

The E-test method, whilst relatively simple, remains a novel method for synergy testing and is not in general use within routine laboratories (Balke *et al*, 2006) and there is some disagreement whether this situation should change in the future (Saiman, 2007). In a small number of studies, however, E-tests have been shown not to agree with previously used methods, though a number of possibilities exist that may cause these discrepant results. The checkerboard method demonstrates inhibition of bacterial growth using a microtitre plate assay with synergy being calculated in a similar way to that of E-testing (*Anonymous*, 1996; Orhan *et al*, 2005; Pankey and Ashcraft, 2005). The time-kill method, however, measures the quantitative extent of killing. Both methods have some detractors and are time consuming (White *et al*, 1996). Other limitations include the relative concentrations of a given antibiotic along with the quality/quantity of inocula. If the concentration of the given antibiotic does not affect the growth curve, then it is difficult to determine whether any effect is additive or synergistic and this can only be determined by the use of a standardised equation that may be seen as being subjective (Moellering, 1979). In addition, the drug concentration is fixed in the time-kill assay. *In-vivo*, the concentration would steadily fall. Combined with the lack of standardised concentrations, this produces yet another limitation. Re-growth of organisms is a possibility where sub-inhibitory concentrations are selected. This, again, can skew the results in any type of broth culture (Pankey *et al*, 2005). It is well documented that where a checkerboard assay is carried out, the results would need to be confirmed by time-kill assay (Petersen *et al*, 2006). The generally good agreement between E-test synergy and that seen using both checkerboard or time kill assays has lead a number of investigators to use the E-test method for determining synergy and antagonism. It is true to say that the checkerboard and very time consuming time kill assays together do show both synergy and the trend for it over time (when combined) – giving a true reflection of the

extent of any synergy. It was also suggested by Petersen *et al* (2006) that the checkerboard assay alone, may overestimate synergy where a bacteriostatic agent is tested in combination with a bactericidal agent.

Table 4.1: Benefits and disadvantages of various synergy test methods (adapted from Saiman, 2007)

	Benefits	**Disadvantages**
Checkerboard Dilution	Easy, uses clinical concentrations	Tests bacteriostatic activity
Multi Combination Bactericidal Testing	Tests Bactericidal activity	May not reflect concentrations in vivo, may not correlate clinically
Time-Kill	Tests bactericidal activity CLSI defined method	Time consuming, tests limited no of agents
E-Testing	Commercially available, can be performed routinely	Bacteriostatic concentrations

E-testing, however, is not only less time consuming, it is also far more practical in the routine laboratory and warrants further use (Principe *et al*, 2009). The advantages and disadvantages of each method used for synergy testing can be seen in Table 4.1 (adapted from Saiman, 2007)

The E-test synergy method uses a concentration that is equal to the MIC for each drug. This MIC matching demonstrates, clearly, any additive/synergistic effect – or, indeed, any antagonistic behaviour. It also allows, on solid media, the scientist to view any resistant sub-populations. This is not possible in the time-kill assay, as it uses a liquid culture system. Pankey *et al* (2005) found that by using the combination elipse effect of the E-testing method, the results were much easier and more practical to review visually. This is true of low MIC synergy that may be more difficult to detect in the checkerboard assay. Where organisms are sensitive to the antibiotic in a broth culture, then synergy cannot be detected.

The E-test method, using solid media, means that even in this situation, the user can view the differences in MIC (Principe *et al*, 2009).

E-testing has been found to be practical, consistent and relatively cheap when compared to other tests. It shows very good concordance with other methods and, whilst still a novel method for synergy testing, is likely to become the *de-facto* standard method for future work (Manno *et al*, 2003; Pankey *et al*, 2005; Petersen *et al*, 2006; Saiman 2007, Principe *et al*, 2009)).

4.1 Clinical relevance of synergy testing

The rationale behind synergy testing is a complex one. One of the non-clinical issues is the lack of drug development vs. the emergence of bacterial resistance. The high cost of development of antimicrobials prohibits their development as pharmaceutical companies only reap a low rate of financial gain when compared with other products (Saiman, 2007). Clinically, it is generally undesirable to use more antibiotics than is absolutely necessary – not just for cost implications, but also to reduce toxicity to the patient (Eliopoulos & Eliopoulos, 1988). However, it has been demonstrated in some cases that antimicrobials may have a positive clinical outcome even where they are noted to be resistant *in-vitro* (Denton & Kerr, 1998; Saiman, 2007). For this reason, synergy testing and the resultant combination therapy may be another tool that brings about successful clinical outcomes. Where combinations are antagonistic, however, then treatment failure may be likely and synergy/combination testing may be able to prevent these combinations from being used (Manno *et al*, 2003).

Antibiotic resistance is a difficult term to define since it is almost entirely dependent upon the dosage that can be tolerated *in-vivo* without overbearing toxicity (Smith *et al*, 2003). Those organisms with an MIC above this level may be termed as resistant, whereas those with an MIC below it may be termed sensitive. It may often be found that the MIC of one

particular infecting organism will also be sufficiently high, that it exceeds that of other organisms or strains of the same organism that are present. The net result of this is that the other organisms/strains may be eradicated, adding a selective pressure towards further resistance. Where multiple combination therapy is used, this problem can be prevented by effectively taking a multi-pronged approach that prevents the emergence of a single resistant strain. A definition of CF related multiple resistance was made by the CF Foundation Microbiology and Infectious Disease Consensus Conference in 1994. It stated that multiple resistance was the "resistance of a bacterial organism to all agents in two of the following classes of antibiotic: the β- lactams, the aminoglycosides or the quinolones" (Aaron, 2007). It should also be noted that of the many studies that have been carried out to test antibiotic resistance – both individually and in combinations, many use isolates from different sources – for example, some studies select their isolates on the basis of multi-resistance, others on invasiveness or other markers. This can skew results, with the end product being a number of studies that are seemingly trying to achieve the same goal but differing in their outcomes and opinions (Bonacorsi *et al*, 1999).

Susceptibility testing is long established in the microbiology laboratory. This testing, however, is almost exclusively aimed at single antimicrobial agents and is indicative of monotherapeutic success. Where organisms such as *Burkholderia cepacia* are involved, it is common to see pan-resistance – rendering single antibiotic testing methods obsolete (Aaron *et al*, 2005). Indeed, single antibiotic testing may not be representative in cases of CF as it will show that most, if not all, antibiotics are resistant. Combination testing may be the only useful way of demonstrating antimicrobial activity *in-vitro* and thus informing future management of these vulnerable patients (Aaron *et al*, 2000). Concomitant use of multiple antibiotics is common in cases of multiple resistance – especially colonisation by Bcc and other colonisers such as *P. aeruginosa*. Studies have demonstrated that where the additive

or synergistic effects of antimicrobials occur, the clinical outcome may be improved – though it is difficult to separate the two effects (Bonacorsi *et al*, 1999).

It has been demonstrated in past studies that synergistic combinations of antibiotics can have a beneficial effect. In conditions such as endocarditis (*Enterococci*) and instances of Gram negative sepsis (White *et al*, 1996) certain groups of antibiotics, such as combinations of β-lactam and aminoglycosides, have repeatedly been shown to improve outcomes – giving enhanced therapy (Eliopoulos & Eliopoulos, 1988; Smith *et al*, 1999).

A study carried out in 2006 by Balke *et al*, whilst not testing *in-vivo*, found that synergy *in-vitro* was not an independent phenomenon. Strains of *Pseudomonas aeruginosa* that demonstrated synergy between two antimicrobials (in this case between ceftazidime and tobramycin), would also correlate with other strains that were found to behave in the same way when tested with the same combinations. In short, the synergy was re-producible with unrelated strains (but not necessarily between species - see below). This may, however, be more of a problem with CF isolates of Bcc spp and other non-fermentative multi resistant Gram negative bacilli, due to the presence of multiple morphotypes and their proven ability to differ in a seemingly random way in their antimicrobial susceptibilities (Foweraker *et al*, 2009).

Whilst the findings of previous work demonstrate that MIC testing in the routine laboratory is an excellent predictive indicator of likely clinical outcome in most organisms, there are notable exceptions – including many of the organisms seen in CF patients (Smith *et al*, 2003). The relevance of antimicrobial testing in CF patients, therefore, has been questioned in a number of studies (Smith *et al*, 2003; Aaron *et al*, 2006; Etherington, 2007; Aaron, 2007, Fowraker *et al*, 2009; Keays *et al*, 2009; Fowraker *et al*, 2010). It has been demonstrated that environmental colonisers such as *Pseudomonas* and *Burkholderia* are often present in multiple morphotypes – each with a different antibiogram. It is very difficult, therefore, to obtain consistent and repeatable MIC data for these organisms as each

subsequent testing may select another colonial variant that, to the microbiologist, visually appears to be the same as the rest (Doring *et al*, 2000; Smith *et al*, 2003; Fowraker *et al*, 2010) giving variable results that may mislead the clinician.

One of the most recent studies to investigate this variability found that *P.aeruginosa* was represented by a mean four morphotypes per sputum sample – and three antibiograms per morphotype. This huge variability makes CF sensitivity testing very challenging, and the interpretation of sensitivity patterns almost impossible (Fowraker *et al*, 2010). This variability in susceptibility testing along with unpredictable outcomes brings into doubt the clinical relevance of such testing in this group of patients. Whilst a number of different methods of testing are recommended (such as combining morphotypes in a single susceptibility test, or testing each individually), it was found that no single method was able to produce reliable results that correlated directly – resulting in an underestimation of bacterial resistance patterns (Fowraker *et al*, 2010). A number of studies where testing and outcome have been compared have found no correlation – including a study carried out in 2003 by Smith *et al*, using *Pseudomonas aeruginosa* as the model organism. This was a retrospective study using patients from the placebo group of a phase 3 trial of nebulised tobramycin. Clinical outcomes were measured using the FEV1 and spirometry both before and after intervention. Their results found that there was no statistical correlation between susceptibility results *in-vitro* and clinical outcome.

This was not the first study to draw these conclusions. An earlier study by Peterson & Shanholtzer (1992) also found similar deficiencies in the correlation between *in-vitro* testing and the clinical outcome. In their study, an animal model was used where a membrane was inserted that would restrict the flow of natural immune factors and other potential confounders. This meant that the MBC *in-vivo* could be tested with some accuracy. They, with a number of other previous studies, found that it was difficult to predict the outcome by use of *in-vitro* testing. Additionally, they found that different sites and origins of pathogens

would also affect the accuracy of this kind of testing – citing an example of a *Staphylococcus* taken from a foreign body in a thigh wound. It was found that the organisms from the foreign body were more resistant than the parent organisms when tested *in-vivo*. They commented that ultimately, it is difficult to replicate *in-vivo* the conditions that are observed *in-vitro*. One of the issues that they highlighted was the variation from patient to patient, where any correlation calculated or perceived, would likely differ between patients. Resistance or tolerance of organisms (defined as a bacteriostatic effect where, under normal circumstances the effect would have been bactericidal) further confounds the picture. It was found in a study by Goldman & Petersdorf (1979) that simply changing the medium in which the organisms are tested can influence the phenotypical expression of resistance/tolerance. In their study, the tolerant organism – *Staphylococcus aureus* (when tested in tripticase soy broth) became sensitive when tested in a different medium (Mueller-Hinton broth). This variable expression of resistance can be a major factor when considering the correlation of *in-vitro* testing to *in-vivo* outcomes (Peterson & Shanholtzer, 1992). Studies have been carried out to assess different ways of testing *in-vitro* that may match more closely the *in-vivo* situation in CF patients. One such study was carried out by Keays *et al* (2009) where a biofilm model was used. They found that where patients receive antimicrobials to treat exacerbations, it was unlikely that organisms would be eradicated completely as antibiotics had been tested upon planktonically grown bacteria. Where antibiotics were tested upon bacteria grown in biofilms, the results of antimicrobial testing were notably more positive and where the biofilm model was used to inform clinical management of patients, improved outcomes were observed against *Pseudomonas aeruginosa*, but not against Bcc species. It is clear that more work will be needed to investigate this further, but a number of authors are now beginning to agree that something has to be done to make testing more representative (Saiman, 2007).

Whilst the E-test synergy method has yet to be officially standardised and clinical correlation is unclear, it does appear to be a promising method for the routine laboratory where single organisms are implicated (Pankey *et al*, 2005). There are studies that have found in favour of synergy testing with good correlation to clinical outcomes, but equally the opposite is also true. Ultimately, the outcome of synergy or MIC testing can only be measured in terms of patient outcomes and successful treatment – though in CF, this is somewhat open ended. To this end, it is well documented that the correlation between such testing is unclear – and this is of particular relevance where long term antimicrobial chemotherapy (such as in CF cases) is to be considered (Smith *et al*, 2003). One of the most recent studies where correlations were taken into account was carried out by Foweraker *et al* (2009). This was a small study using *Pseudomonas aeruginosa* as their subject organism, but demonstrated many of the same issues as found with other CF related pathogens – such as polymorphic susceptibilities from apparently pure cultures. Their study found that differing methods of testing actually produced different susceptibilities – further throwing doubt upon the usefulness of MIC and combination testing. Additionally, as they tried to correlate response to their testing, they found that no single method was predictive of outcome. It is difficult to assign the appropriate weight to this study, as it was limited in size – though thoroughly carried out. To this end, it was suggested by Smith *et al* (2003) that whilst a correlation is not detected within their own statistical confidence intervals, this does not conclusively indicate that it does not exist. The possibility of better correlations may exist where larger numbers are used, though this would be particularly expensive and challenging to realise. This, however, was not the only study to carry out such work; a study in 2007 by Etherington *et al* found that a reduction of susceptibility testing had little bearing upon clinical outcomes of IV therapy. Despite this, however, the authors were unable to categorically reject the use of such testing as there were a number of significant areas where this may be useful (for example, identifying unusual isolates such as the Bcc species, and the identification of multi-resistance for infection control purposes).

Clinical failure carries with it a huge risk to the patient. Many clinicians will be aware that such long term treatments will produce resistant bacteria that will make up a percentage of any population – and may ultimately be selected for during treatment. The multiple morphotypes that are thus produced – and are commonly seen in CF isolates, may mislead the clinician as they will often produce widely differing sensitivity patterns leading to uncertain and difficult clinical decisions based on uncertain data. Many clinicians, therefore, will judge clinical failure or success clinically and the results of synergy testing overall are only to be used as a guide to the *potential* of the agent tested (Smith *et al*, 2003; Mariscal *et al*, 2006). It can be difficult to judge, however, whether treatment is warranted with an antibiotic that has been reported as resistant during susceptibility testing. Such isolates, it has been demonstrated, may still respond clinically (Denton & Kerr, 1998; Aaron, 2007). Smith *et al* (2003) pointed out that there are a number of difficulties that may be experienced in assessing whether antibiotics have had the appropriate impact. It is difficult to prospectively carry out such assessments due to the ethical difficulties of withholding or using a treatment where susceptibilities suggest otherwise. The resulting retrospective nature of studies, therefore, do not always account fully for confounding factors. In their study, they point out that their only marker of positive/negative outcomes was an increase or decrease in pulmonary function. Inclusion of other clinical factors such as resolution of coughs or sputum production would provide further guidance but there are still many factors that may be overlooked – such as the timing of treatment (though in the study carried out by Smith *et al* (2003) this was accounted for during their regression analysis), dose regimen and other drugs that are being used concurrently. In their study, Smith *et al* (2003) found that there was no significant trend linking susceptibilities with outcomes (using *P. aeruginosa* as the model organism) though they did stop short at suggesting that no trend existed – only that it may not be clinically significant.

A double blind, randomised, controlled clinical trial to assess the correlation between multiple antibiotic combination testing (MCBT) and clinical outcome, where patients were allocated antibiotics either from the more standard single routine testing method, or from MCBT was carried out by Aaron *et al* (2005). A large number of patients (n = 132) were used at the time of exacerbation and a large range of clinical criteria were used to assess outcome. In summary, this very thorough study found that whilst patients certainly did improve with antimicrobial therapy, there was no difference associated with treatment that was as a result of MCBT. Whilst MCBT differs slightly in its method of testing (using multiple wells and combinations until a successful combination is found that is bactericidal), the outcomes can be considered to be very similar – and correlation with clinical outcomes apply equally to such testing.

Whilst there are a number of studies that suggest no correlation between synergy testing and clinical outcome, there are those that do find in favour of such an approach. In a study carried out by Orhan *et al* (2005), it was found that the synergy results correlated well with both treatment regimen/dosage, and clinical outcome. Petersen *et al* (2006) suggest that despite these problems, where empirical treatment is to be administered, synergy testing may at least provide some guidance as to the likely outcome. At worst, it will prevent the clinician from using antagonistic combinations. This is a position taken by other commentators (Mariscal *et al*, 2006). Work has demonstrated that combinations are anecdotally more likely to deliver improved clinical situations for patients, whilst reducing resistance (Saiman, 2007) and some clinicians are very keen to use synergy testing as a further tool where there are difficult treatment decisions to be made.

The clinical relevance of susceptibility testing in CF patients – regardless of method used, remains a divisive and unresolved issue and is the subject of much discussion.

4.1.1 Previous relevant synergy studies with tigecycline *in-vitro*

A number of studies have addressed the novel glycycline, tigecycline and its potential to exhibit synergy when combined with other antimicrobials. A review of the literature reveals a number of articles citing tigecycline as a potential novel antibiotic agent for use against resistant non-fermentative Gram negative bacteria such as *Acinetobacter spp* (Jones *et al*, 2007), but these are rarely CF isolates and no rigorous testing of MIC has been carried out to date in the Bcc or other unusual isolates (Denton & Kerr, 1998; Milatovic, 2003). A large study was carried out and tested tigecycline against a large panel of non-CF organisms – including environmental strains of *S. maltophilia* (Entenza & Moreillon, 2009). They found some synergy (amikacin/tigecycline and colistin/tigecycline), though the net result was indifference. No antagonism was found.

A feature of many of the studies that, in particular look at the more unusual isolates, is their relatively small sample numbers. This can make it difficult to apply study results to other clinical situations. This is particularly the case in CF which is a very specific disease with its own specific clinical problems. One reason for the low numbers, however, is simply the lack of availability of isolates from relevant clinics. By their very nature, the isolates are unusual and, therefore, relatively uncommon. As such, one must use comparisons with some care but some of these studies may be predictive of outcomes in further work as stated previously.

One study in 2009 by Principe *et al*, used only twenty two isolates of (MDR) *Acinetobacter baumanii*. They tested levofloxacin, piperacillin-tazobactam, amikacin, imipenem, rifampicin, ampicillin-sulbactam and colistin using the checkerboard method rather than the solid culture E-test method used in this study. Some synergy was observed – most notably, was that of colistin (8.3%), amikacin (8.3%) and imipenem (8.3%).

Demonstrating that these results are likely to be species specific, however, are other studies that have found either no synergy or very little synergy when testing with other species. One such study (again using relatively low numbers) was carried out by Petersen *et al* (2006). They found that some synergy between amikacin and imipenem was demonstrated against *Proteus* species. No synergy was found when tested against *Stenotrophomonas maltophilia* but equally, antagonism, in common with the studies that have been carried out, was practically non-existent. Again, it is difficult to judge the relevance of such a study particularly as they used a wide range of organisms but with relatively low numbers of each type. When testing piperacillin/tazobactam in combination with tigecycline, 2/3rds were found to show synergy. The problem here being that there were only three isolates tested in this capacity. Piperacillin was tested alone (without tazobactam) and 3/11 isolates were found to demonstrate synergy. Again, comparing this with the 2/3 isolates tested against tigecycline and piperacillin/tazobactam is difficult and any comparison with other studies or clinical outcomes must be done with some care. Antimicrobials tested in this capacity against *S. maltophilia* did not demonstrate any synergy (Imipenem, piperacillin – both 0/11 isolates). By way of direct comparison, as much as this is possible, with the study by Principe *et al* (2009), colistin (1/9), amikacin (4/9) and imipenem (3/11) were all found to demonstrate synergy against *Acinetobacter* species when in combination with tigecycline. This shows some concordance with the previous study, but again backs up the likelyhood that synergy is likely to be species specific. Interestingly, none of the strains in the Petersen study showed antagonism – demonstrating only synergy or indifference (Petersen *et al*, 2006). *S. maltophilia*, in recent years, has been the target of a number of studies and is a difficult problem due its resistance mechanisms. Synergy studies have suggested that multiple combinations may be more effective than simple pairs. Additionally, higher concentrations have not surprisingly, been found to be more effective. Colistin is noted to be more effective than tobramycin or gentamicin when tested at higher concentrations (Gabriel *et al*, 2004). The study carried out by Gabriel *et al* in 2004 demonstrated high

levels of synergy between a number of groups of antibiotics – some of which are included in this study. Whilst they did not test tigecycline, doxycycline was used and was noted to be effective in combination with ticarcillin clavulanate (49% of strains tested). It is difficult from this study to determine the exact route of synergy as they used not only antibiotics, but also β-lactamase inhibitors that would further destroy or inhibit resistance to these antibiotics. It was interesting to note the high levels of resistance that the study found (78% multiresistant strains) but also that 91% of the isolates tested demonstrated synergy and susceptibility to at least one combination that was tested. How practical it would be to routinely test all of these combinations remains an issue to be resolved (Saiman, 2007).

A multiple organism study carried out in 2007 by Vouillamoz *et al*, found that most reactions between tigecycline and other antimicrobials demonstrated indifference. Whilst this was a promising result in terms of drug-drug interactions, it did mean that the additive or synergistic effect of other antimicrobials may be negligible *in-vivo*. Where limited amounts of synergy occurred (for example between tigecycline and co-trimoxazole versus *S.maltophilia*), they were found to be of an uncertain and possibly non-specific nature across species. This makes interpretation of such interactions difficult – especially when trying to correlate this to clinical outcomes.

A study by Petersen *et al* (2006) failed to observe any antagonism where tigecycline was tested in combination with other antimicrobials (including some of those tested in this study). Petersen *et al* (2006) found that synergy occurred when tigecycline was combined with minocycline. Petersen *et al* (2006) found higher levels of synergy in their panel of Gram negative bacteria (for example, amikacin 56%; piperacillin/tazobactam 50%). Given that this panel of bacteria did not include the Bcc species, it is difficult to directly compare these results. They did, however, test *S. maltophilia*, and it should be noted that no synergy to their combinations was observed in this case. The study was marred a little by low numbers, but may demonstrate that where organisms are in possession of multi resistance

mechanisms, the effect of synergy may be effectively diluted. This may explain why previous studies have observed these "higher" levels of synergy. Alternatively, however, one cannot discount the effect of low numbers. In our group of "other" Bcc species, we had similarly low numbers which meant that a single organism showing synergy would disproportionately skew any results given.

Aaron *et al* (1999) carried out a large ranging study against *Burkholderia* isolates. Antibiotics, were tested individually and in combinations – including up to triple combinations. They found that the most effective antimicrobials against *Burkholderia* were ceftazidime, meropenem and high dose tobramycin. In double combination, the most effective pairs were meropenem-minocycline, meropenem–amikacin, meropenem-ceftazidime, meropenem-tobramycin and tobramycin-ceftazidime. The antagonistic interactions of the antibiotics in this study demonstrated the extent to which this can occur. Antagonism where meropenem, ceftazidime and tobramycin had a second antibiotic added was fairly widespread – ranging from 65% with ceftazidime to 31% with meropenem or tobramycin. The study did demonstrate, however, that the addition of a third antibiotic could negate this antagonistic reaction. Where triple antibiotics were tested in combination, these were often the most effective.

4.2 Aim and objectives of the project

4.2.1 The setting

The Leeds Teaching Hospitals NHS Trust runs the second largest CF Centre in the United Kingdom – covering a large population and geographical area. The Centre cares for children and adults with CF from a catchment area covering the North, East and West of Yorkshire, including ten district general hospitals. The service provided by the CF units are fully integrated with other specialist departments such as paediatric intensive care, transplant wards and also oncology units. The clinics and wards offer both in-patient and out-patient referrals and are among the best equipped in the UK.

It can be very difficult with the unusual isolates found in CF patients, to select an empirical therapy. Courses of antimicrobials are relatively expensive and are prone in some cases, to failure. The multiple resistance factors possessed by many of these organisms mean that resistance rates are high and susceptibility cannot be reliably predicted. Combination therapy is therefore frequently used, and this study aims to look at tigecycline in combination with other agents so that it might inform clinicians in their future selection of treatment (Aaron *et al*, 2000). Indeed, previous randomised, placebo controlled trials have shown that combination therapy is more effective against resistant colonising organisms such as *Pseudomonas* species (Aaron *et al*, 2000).

The overall aim of this project was to ascertain the *in-vitro* activity of tigecycline against clinical strains of multi resistant Gram negative bacilli (including *B. cepacia* complex, *S.maltophilia* and others) isolated from CF patients.

4.2.2 Specific objectives

1. To identify, from a 10-year retrospective collection of multi-resistant isolates from CF patients, duplicate organisms (and possible epidemiological clusters) using PFGE.
2. To obtain the MIC of tigecycline and other commonly used antibiotics against a panel of clinical strains isolated from CF patients over a 10 year period.
3. To demonstrate the interaction between tigecycline and other commonly used antibiotics that are used to treat CF patients, using an *in-vitro* synergy model of E-testing and fractional inhibitory concentration calculation.
4. To improve the holistic service given to CF sufferers within the Leeds Teaching Hospitals NHS Trust. This will be a direct result of the fuller understanding of the efficacy of tigecycline, both alone and in combination, in the treatment of patients with chronic colonization.
5. To inform our local knowledge of the epidemiological spread of these organisms. Resistance patterns, in conjunction with the epidemiological information will further inform clinicians and their treatment of CF patients.

These objectives were achieved by the scientific calculation and testing of MIC data, and the combination testing of organisms/antibiotics taken from the 10 year collection. It is difficult to judge the clinical outcomes and relevance of such data, due to the poorly understood correlations between synergy/MIC testing and clinical outcome. However, this data should give an indication as to the likely clinical success and will provide information that may be useful for clinicians that treat CF patients on a daily basis.

4.2.3 Ethical approval

Ethical approval was applied for and granted by the Leeds Research Ethics Committee (REC reference number: 08/h1307/42). The full response from the LREC can be obtained in Appendix 1.

Chapter 5

5.0 Materials and methods

This study was carried out on isolates gathered over the last ten years. These were principally from patients attending the Regional Paediatric and Adult CF Units in Leeds. The isolates were identified in the Microbiology Department of Leeds Teaching Hospitals NHS Trust (LTHT), with further characterisation of *Burkholderia spp* and others being carried out at the Centre for Infections, Health Protection Agency, Colindale, London, UK. Isolates were identified using a number of techniques including 16s PCR and fatty acid analysis. Laboratory type strains were obtained from EuroCare CF (University of Ghent, Belgium) and run as controls. Following identification and purification, all of the isolates were stored at -80^{o}C in 15% glycerol broth. Upon testing, a small number of historical isolates were irretrievable and were therefore excluded from the study.

Table 5.1: Final collection of isolates used in this study

ISOLATE	NUMBER TESTED
Burkholderia multivorans	31
Burkholderia cenocepacia	16
Burkholderia stabilis	2
Burkholderia viatnamiensis	1
Burkholderia gladioli	1
Achromobacter xylosoxidans	20
Stenotrophomonas maltophilia	47
Chryseomonas indologenes	1
Chryseomonas meningosepticum	1
Delftia acidivorans	2
Pandorea apista	1
Pandorea sputorum	1
Pseudomonas stutzeri	2
Shewanella putrefaciens	2
Total	**128**

When drawing conclusions from this work, it should be noted that over this 10 year period, these isolates will have recurred in the same patients as they have become colonised with them. Duplicate isolates from the same patients, therefore, were not included in the study as they would yield no further, relevant, information. Additionally, it is not the policy of the LTHT Pathology Department to store duplicate isolates, and so only a prospective study could address the extra numbers of organisms that would have been required for these to be added.

All of the strains used in the study had been stored in glycerol broth at -80^{o}C. The isolates were sub-cultured onto Columbia blood agar (Oxoid) and incubated at 37^{o}C for 24 to 48 h (depending upon species). This allowed us to ascertain the purity of these stored organisms. All of the organisms used in this study were then re-stocked in glycerol broth for future use. Any organisms that were to be used were re-cultured in the same manner as described above. The final collection of strains is listed in Table 5.1 above.

5.1 Pulsed field gel electrophoresis

Given the scope of the collection, in that it was obtained in a geographically related population, it was prudent to type these strains in order to eliminate any duplicates by way of relatedness. Pulsed field gel electrophoresis (PFGE), therefore, was used as it is arguably the best method for interpreting a wide array of strain types (Tenover *et al*, 1995). There are likely to be inaccuracies in any system and with bacteria such as those tested in this study, point mutations and insertions are a potential confounding factor – often leading to variable results. Using Tenover's criteria, however, this system can be reasonably accurate for predicting relatedness. Where greater than ten bands exist on the PFGE fingerprint, the PFGE profile is considered to be accurate. The following criteria proposed by Tenover *et al* (1995) were used:

Indistinguishable: Related isolates that are likely to be indistinguishable even when using other techniques. This is where all of the bands correspond with each other with no distinguishable difference.

Closely related: A closely related isolate will differ by only a single genetic event. Only 2-3 band differences would be seen in such circumstances where a point mutation or insertion has occurred that will create a new restriction site. Variations such as this are occasionally seen when a single isolate is frequently re-cultured or re-isolated from the same site/patient.

Possibly related: Where two genetic events have occurred, then a difference of around 4-6 bands will be seen. This could be explained by the gain or loss of restriction sites resulting from two deletions or insertions. These strains would be considered likely to be from the same lineage but not necessarily epidemiologically linked.

Unrelated: A difference of three or more genetic events will render an isolate different by at least seven bands. Using Tenover's assumption of approximate ten fragment accuracy, this would represent less than 50% similarity by fragment presence.

Given the criteria stated above by Tenover, a bionumerics programme was used to calculate "relatedness" and the results are presented in Figures (6.1–6.4) respectively. These comparisons were not made for groups such as the "other CF related Gram negative bacilli" (GNB) as these groups contain mixed species for information only and any comparison would be meaningless.

5.1.1 Method for typing by pulsed field gel electrophoresis

Day 1

Strains were inoculated into 5 mL of tryptone soya broth and incubated for 6 h at 37°C with shaking to attain exponential growth. They were then pelleted by centrifugation at 3,000 x g for 10 min, and the supernatant discarded (Columbia blood agar purity plates were cultured

at this stage). The cells were then re-suspended and washed in SE buffer (25 mM EDTA [pH 7.4], 75 mM NaCl) before re-centrifugation. Where mucoid strains were encountered, the process was repeated. After the addition of 0.3 mL EClysis, the cells were again vortexed before the addition of 0.3 mL low melting point agarose. This was gently mixed using a Gilson pipette. This mixture, whilst still warm, was pipette into plug moulds (10 by 5 by 1.5 mm), allowing time to set at 4^{o}C. The agarose blocks were then removed to bijoux bottles containing 1 mL of 1 mg/mL proteinase K in EClysis and then incubated overnight at 55^{o}C.

Day 2

Purity plates were checked at this stage – if mixed, the process proceeded no further until organisms had been purified.

EClysis was removed from the bijoux containing the agarose blocks. It was then replaced with 3 mL TE buffer. The blocks (in bijoux) were then placed onto a roller and washed for 30 mins. The buffer was then replaced, and the process repeated for a further four times. After this, the bijoux containing agarose blocks and TE buffer, may be stored at 4^{o}C until required.

Day 3

Bijoux containing blocks for testing were removed from refrigeration and were washed again with fresh TE buffer (3 mL for 15 min). A block was then removed and cut to size (approx 1 mm width) and placed into an Eppendorf tube (gloves should be worn throughout this process). They were then placed in 750 mL of restriction digest buffer H (50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol [pH 7.5]) and washed again for 15 min with rolling. Fresh H buffer was used to replace the previous buffer, SpeI restriction endonuclease (Boehringer, Mannheim, Germany) was added, and plugs then incubated at 37 °C for 6 h. SpeI was used as this cuts clearer restriction fragments and is the recommended restriction enzyme for *Burkholderia* species (Anderson *et al*, 1991; Tenover *et al*, 1995).

Whilst this process is ongoing, a fresh TBE Buffer was prepared. One g PFGE agarose was then added to 100 mL TBE Buffer. After melting in a microwave (1 min followed by 30 s bursts) the resulting mixture was left to cool slightly. During this phase, the pre-prepared blocks were added to a gel comb and the locations recorded. The PFGE agarose was then poured into the gel cassette and left to set for approximately 30 min. The TBE buffer was added to the PFGE tank and cooled to 14^{o}C.

Electrophoresis was performed using the contour-clamped homogeneous electric field DRII system (BioRad Laboratories) over 21 h at 14°C with 5 to 35 s of linear ramping at 6 V/cm. Lambda ladder was incorporated as a size standard along with a number of laboratory strain controls. Electrophoresis products were visualized by ethidium bromide staining, and patterns compared by eye and a gel documentation system. Interpretation was based on the criteria of Tenover *et al* (1995).

5.2 Determination of MIC

Once multiple isolates had been removed, then the remaining collection was subject to MIC determination (Pankey & Ashcraft, 2005). This was carried out by the use of E-testing (method in accordance with manufacturer's guidelines, AB Biodisk, Solna, Sweden).

MICs were determined on the following antibiotics: aztreonam, amikacin, ceftazidime, ciprofloxacin, colisitin, meropenem, minocycline, piperacillin-tazobactam, temocillin, tigecycline, tobramycin. These antimicrobial agents were chosen for their potential chemotherapeutic relevance to CF patients. Other antibiotics noted to be successful against the organisms were not necessarily relevant to this study – for example chloramphenicol. Whilst useful against *S. maltophilia*, it is noted that many patients at risk of infection from this organism, also have other haematological risk factors and thus would be at risk of severe further complications from using this antimicrobial (Denton & Kerr, 1998).

5.3 *In-vitro* E-test synergy testing

Synergy testing was carried out using the method described by Pankey & Ashcraft (2005). A combination of two E-test strips was used and the test was performed in duplicate to ensure sufficient rigor from which to draw conclusions.

Briefly, the method used involved fresh cultures of the target organisms. The organism was lawned evenly onto plain Muller Hinton agar (OXOID) and the first strip applied. The MIC of the organism to this particular strip was marked on the agar using a straight wire, and the strip was left to incubate at room temperature for 1 h in order to ensure sufficient diffusion of the antimicrobial agent.

After 1 h, the strip was removed and replaced with the second antimicrobial. The MIC (previously determined) of this strip was lined up to match that of the previously applied strip. The rationale behind this being that the resulting ellipse (where indifference is observed) should line up precisely at the previously recorded MICs for the target organism. Where additive or synergistic results were seen, the ellipse would have moved to a lower point of the strip than that previously recorded. The strips were applied in the reverse order during repeat testing to eliminate any experimental bias.

The net result of this testing method was to determine an *in-vitro* MIC based upon the use of tigecycline in combination with other antibiotic agents. It identified where synergy occurs (increasing its effectiveness), and where antagonism occurs (this will decrease the effectiveness of tigecycline in combination with these antimicrobial agents).

Tigecycline was tested in combination with the following antibiotic agents: aztreonam, ceftazidime, ciprofloxacin, colistin, meropem, piperacillin-tazobactam, temocillin tobramycin.

Minocycline and amikacin were not tested further as minocycline is an analogue of tigecycline and would not currently be used combination. Amikacin has been demonstrated in previous studies to be less effective than tobramycin (this was also found during MIC testing) and in order to cut down duplication of antibiotic groups, tobramycin was used to represent the aminoglycoside group (Bonacorsi *et al*, 1999).

The summation fractional inhibitory concentration (FIC) was calculated for each set of MICs using the recommended method by the Journal of Antimicrobial Chemotherapy (ANON 1999) and other authors (Pankey & Ashcraft 2005, Saiman 2007).

The calculation of FIC was by the following method:

FIC of drug *a* = (MIC drug *a* in combination)/(MIC drug *a* alone)

FIC of drug *b* = (MIC drug *b* in combination)/(MIC drug *b* alone)

ΣFIC = FIC of drug *a* + FIC of drug *b*.

Synergy was defined by a ΣFIC of ≤ 0.5

Antagonism was defined by a ΣFIC of > 4.0

Interactions represented by a ΣFIC of > 0.5 but ≤ 4.0 were to be termed indifferent (Pankey & Ashcraft, 2005).

5.3.1 A brief note on calculation of synergy

An inevitable question will be raised regarding the calculation of synergy, and indeed the validity of the calculations. Whilst it falls outside the scope of this study to devise the optimum method of calculation, we did note that this calculation is a seemingly arbitrary method of determining synergy. The difficult question in this situation is to ask "what actually defines synergy" – a quantitative measurement that to one individual may mean something entirely different than that to another. In the case of the calculations used,

synergy is loosely defined as a four-fold reduction in the MIC of each agent alone, when compared with that in combination (i.e. the combination is more efficacious). As mentioned previously, there are a number of different ways of expressing this data, but ultimately, these all stem from the same calculations and use the same cut-off points. To this end, we did attempt to determine the origin of the calculations. We noted that the trail ended in most journal articles, at the Journal of Antimicrobial Chemotherapy (Anon, 1999) where the equation was published under an anonymous author as part of their guidelines for submission of scholarly articles. All of the other papers that we reviewed as part of this study either independently used the same calculation without reference to previous work, or referenced the article above. Finally, we noted a journal article that compared a number of different calculations, (Saiman, 2007) with the result that we deemed that this calculation was valid for our purposes. It should be noted, that in favour of this decision, is the fact that all other authors submitting work to respectable journals such as Journal of Antimicrobial Chemotherapy, are using the same method. To this end, we can be reassured that the work is standardised and comparable to other work published in this field.

Chapter 6

6.0 Results

6.1 Typing by pulsed field gel electrophoresis

The pulsed field gel electrophoresis (PFGE) demonstrated that whilst there was some clustering of isolates, they were all generally unrelated. It has been demonstrated in multiple studies that whilst these organisms may have been isolated in the same CF clinic, it is almost impossible to tie them together epidemiologically and for this reason, no isolates were excluded from the study on the basis of the PFGE results alone (Denton & Kerr, 1998; Andersen *et al*, 2007). A small number of isolates were untypeable by PFGE despite repeated attempts. These, therefore, also remained in the study.

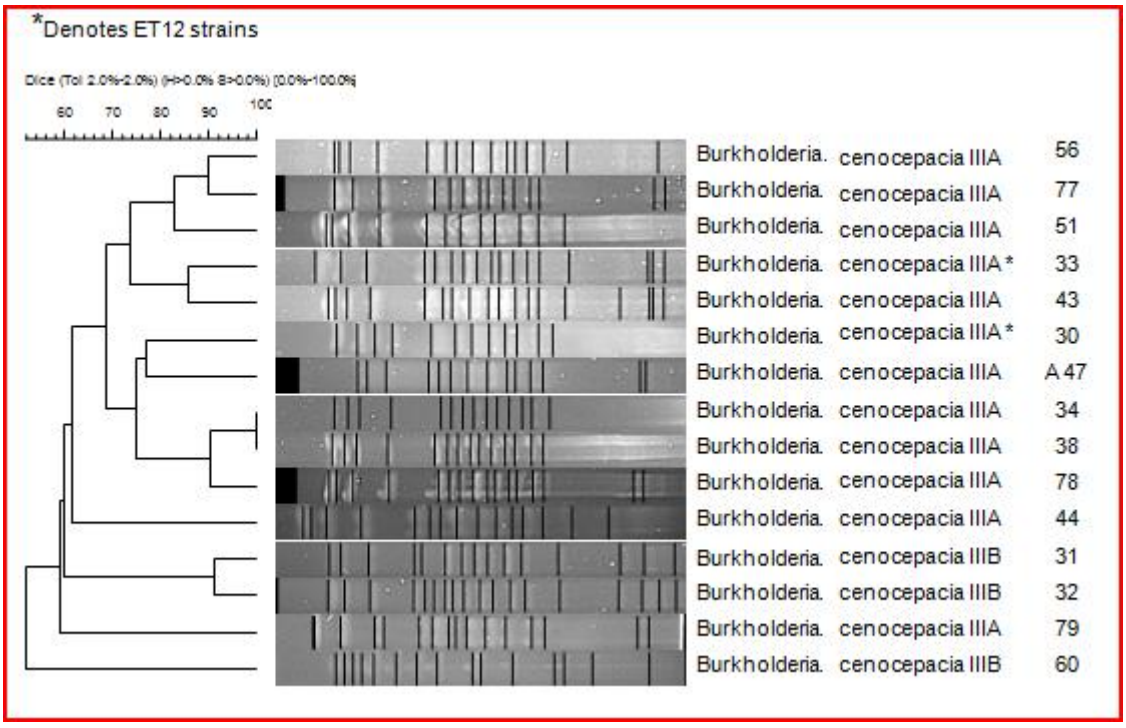

Fig 6.1: Pulsed field gel electrophoresis comparison of *B.cenocepacia* isolates

Fig 6.1 shows the PFGE comparison of the *Burkholderia cenocepacia* isolates (number in the right hand columns of each of these figures represent those assigned at their time of storage within the laboratory). This group encompasses the IIIA, IIIB genomovars and,

within these, the ET12 epidemic strain (n = 3 (one of which was untypeable by PFGE and does not appear in Fig 6.1 above)). The second group of isolates that show around 90% relatedness are notable in that they have an average of 4/5 band differences. By Tenover's (1995) criteria, 4-6 bands difference suggest possible relatedness. As with the *B.multivorans* isolates however, there are some subtle differences in MIC for some of these isolates. *B.cenocepacia* numbers "34" and "38" whilst being in the order of 90% relatedness, demonstrated significantly differing MICs almost across the board. As with the *B.multivorans* isolates, it seems prudent to rate these strains as being different from each other. *B. cenocepacia* numbers "31" and "32", however, are the most closely related isolates in this study with approximately 100% relatedness both by bionumerics calculation, and Tenover's criteria. It is significant to note also that the MICs of these organisms do not appear to differ. These organisms were tested further as part of the experimental protocol (since the relatedness of these isolates is not a foregone conclusion) and minor differences were found in the synergy testing. These isolates, therefore, were retained as it was judged that they would not influence, unduly, the final outcome of the trial.

Fig 6.2 shows the comparison of *Burkholderia multivorans* isolates. Using the bionumerics programme, it can be seen that the closest related isolates are isolates "52" and "71" at approximately 90% relatedness. By Tenover's criteria, we would consider these two isolates to differ by six bands meaning that, at best, they are "possibly related". The next nearest pair are around 88% related as calculated by the bionumerics programme. Again, here we see approximately eight bands difference which, by Tenover's criteria, would make these strains "unrelated". These results imply, therefore, that only 12.5% of *B.multivorans* isolates tested are potentially related in some way. Equally, it may be said therefore, that 87.5% of isolates are clearly unrelated. Comparison of MIC data shows that isolates 52 and 71 differ in most of the antibiotics tested, thereby backing up the assumption that these two isolates are, in fact, unrelated.

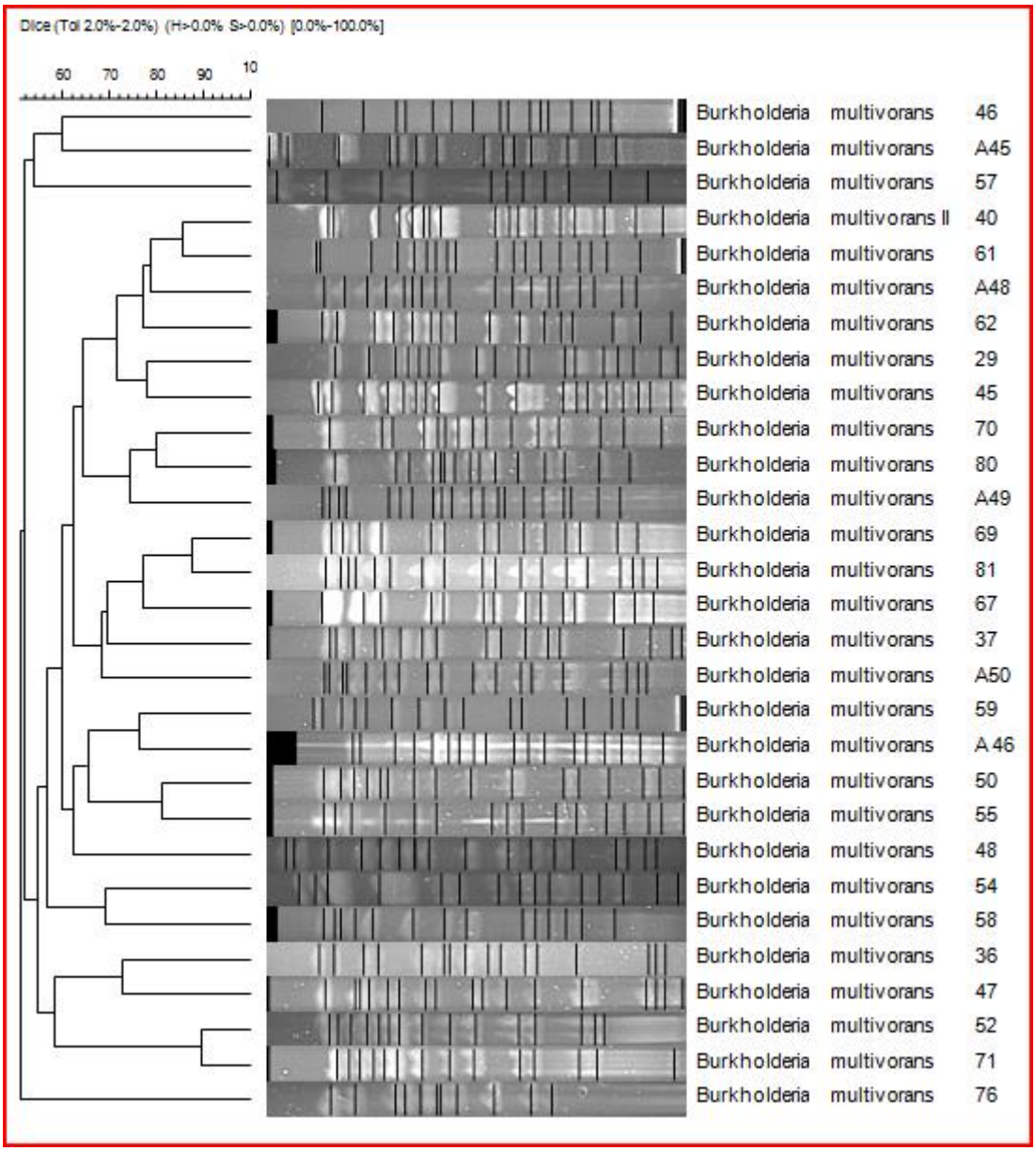

Fig 6.2: Pulsed field gel electrophoresis comparison of *B.multivorans* isolates

As with previous groups of isolates, the *Achromobacter* isolates proved to be largely unrelated – with the closest of the group being in the order of around 88% similarity. One pair, however, did have identical PFGE profiles. This pair were not excluded, however, on account of having differing MICs to a wide range of antimicrobials. As with previously similar isolates, the full range of synergy testing was also carried out and differences between the two isolates were observed (for example, isolate "A9" demonstrated synergy to the colistin-tigecycline combination, whereas "A8" demonstrated indifference).

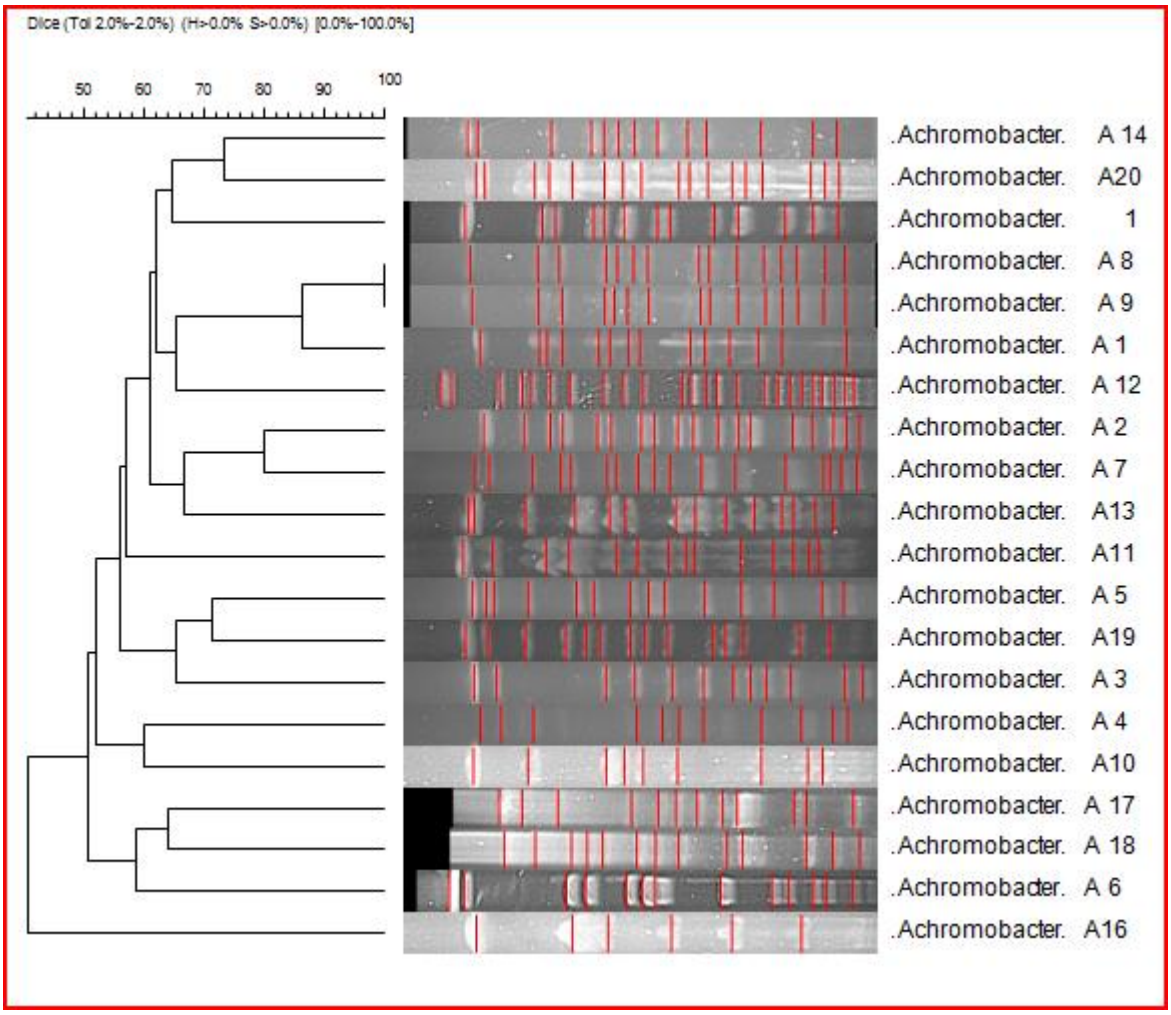

Fig 6.3: Pulsed field gel electrophoresis comparison of *Achromobacter xylosoxidans* isolates

Fig 6.4 shows the PFGE comparison of *S. maltophilia* strains. The vast majority of these strains are clearly unrelated by PFGE. The closest pair here being "S32" and "S33". As with other closely related strains, these two strains demonstrated differing MICs to a range of antimicrobials, and for this reason these strains were retained in the study. The next closest group were classified by bionumerics as around 90% related and by Tenover's criteria, these would be classified as "closely related". Once again, marked differences in MIC were observed during the antimicrobial testing protocol, and these isolates have been retained.

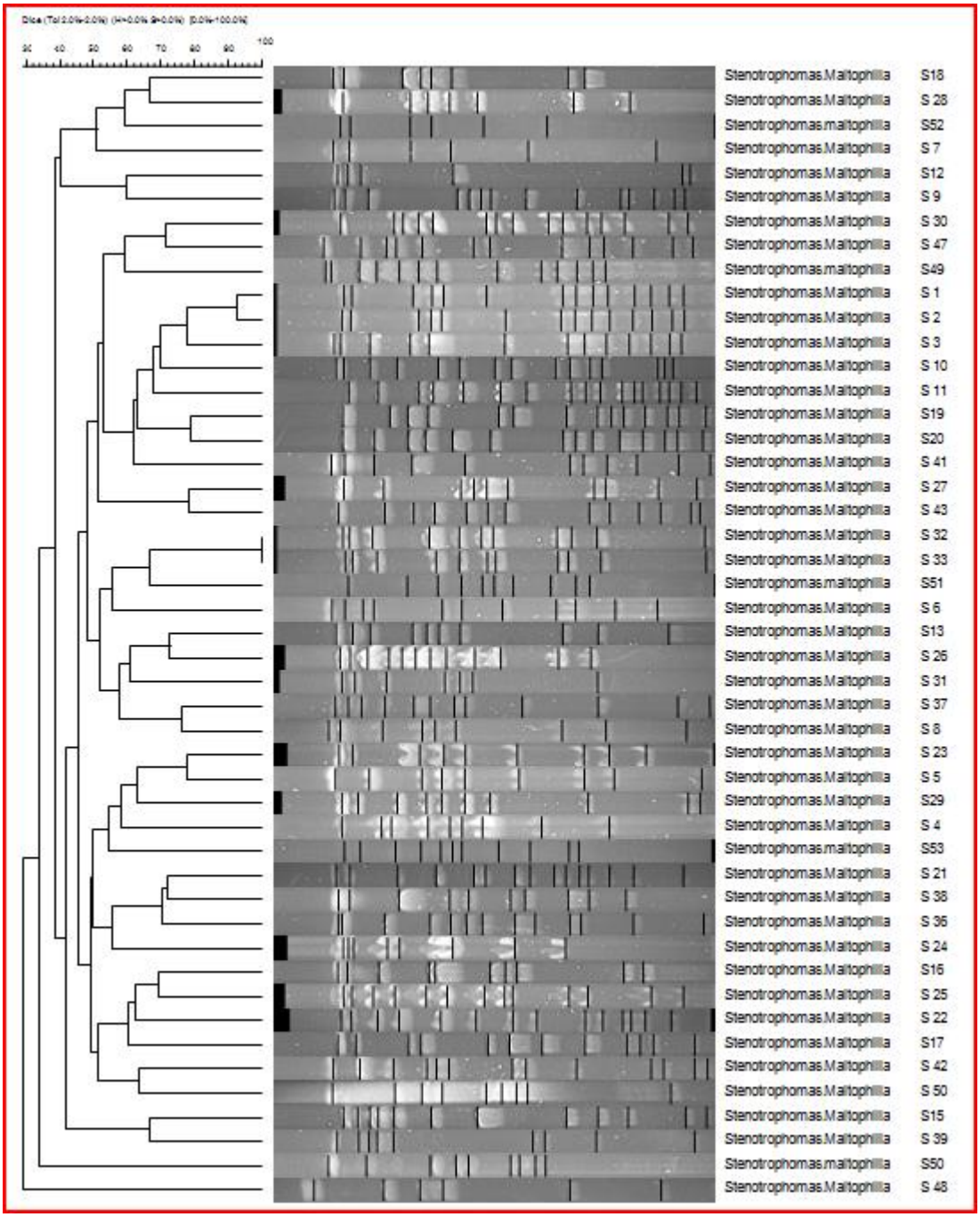

Fig 6.4: Pulsed field gel electroporesis comparison of *Stenotrophomonas maltophilia* isolates.

6.2 MIC/synergy testing

The results of both MIC and synergy testing can be seen in Tables 6.5 and 6.6 respectively. In summary, the most effective antimicrobials observed via MIC testing can be seen in Table 6.1 below:

Table 6.1: Pathogen groups and the most effective antimicrobials *in-vitro* found from our study

SPECIES GROUP	Most effective	2nd most effective
B.cenocepacia	Minocyline	Ceftazidime, meropenem
B.multivorans	Minocyline	Ceftazidime
Other Bcc spp	Minocycline, meropenem, ciprofloxacin	Piperacillin/tazobactam
A.xylosoxidans	Minocycline	Tigecycline
S.maltophilia	Minocycline	Tigecycline
Other CF related GNBs	Minocycline	Tigecycline

^The small group of "other" *Burkholderia* species and "other CF related GNBs" have been included in this section, though it is acknowledged that sufficient numbers are not present to reach a conclusive definition of sensitivity/resistance. This group, therefore, appears for illustrative purposes only.

6.2.1 Synergy/antagonism by species/group

This section has been included to show the occurrence of synergy/antagonism/indifference across isolate groups. A brief check of the data will indicate that there are general trends in the status of the organism (resistant/sensitive) and the occurrence of synergy (Table 6.2). Where organisms were resistant or sensitive to the target antibiotic alone, this was compared with the occurrence of synergy/antagonism when these antibiotics were tested in combination with tigecycline. It would appear that in most groups, synergy occurs where organisms demonstrate resistance to the target antibiotic. Likewise, it would appear that antagonism also occurs primarily in these groups of organisms. The exception to this, are the *S. maltophilia* and "other Bcc" groups.

Table 6.2: The occurrence of synergy and antagonism with respect to original MIC data

ORGANISM GROUP	MIC RESULT	OCCURRENCES OF: Synergy	Antagonism
B. cenocepacia	Resistant	9	0
	Sensitive^	0	1
B. multivorans	Resistant	24	9
	Sensitive^	8	1
"other Bcc"	Resistant	2	1
	Sensitive^	6	0
S. maltophilia	Resistant	19	24
	Sensitive^	2	13
A.xylosoxidans	Resistant	9	0
	Sensitive^	1	0

^ Includes sensitive intermediate organisms.

6.2.2 MIC/Synergy testing data

The following figures have been included to show comparative synergy/antagonism and indifference of each species against combinations of antibiotics. Each figure represents one of the antibiotics tested in combination, and synergy/antagonism/indifference are expressed as a percentage.

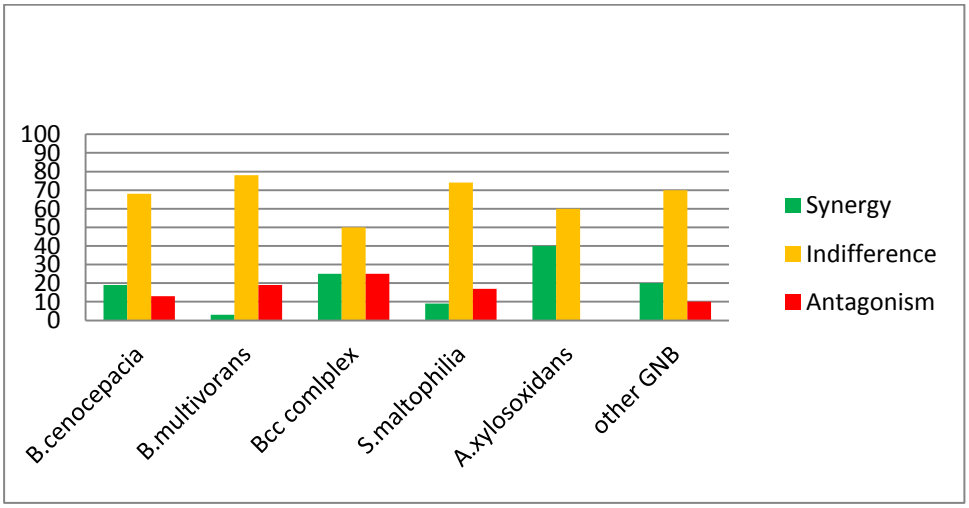

Figure 6.5 Percentage synergy/indifference/antagonism of isolates tested with colistin and tigecycline in combination.

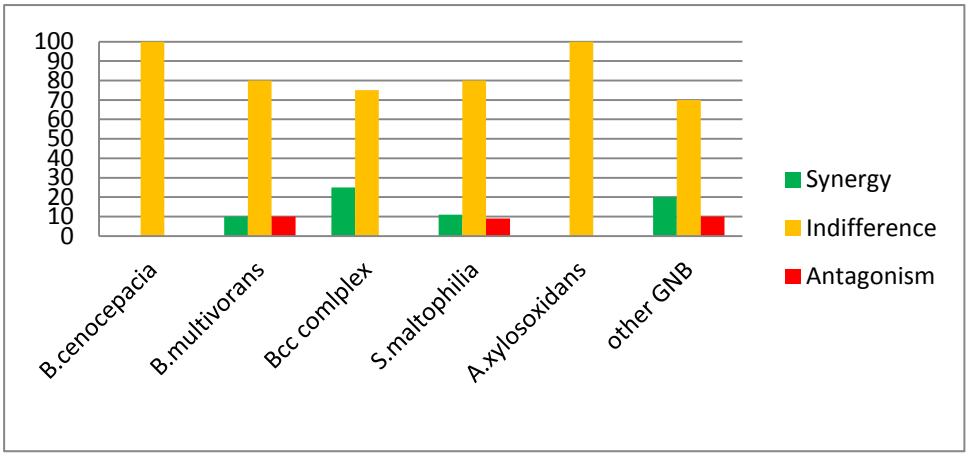

Figure 6.6 Percentage synergy/indifference/antagonism of isolates tested with ciprofloxacin and tigecycline in combination.

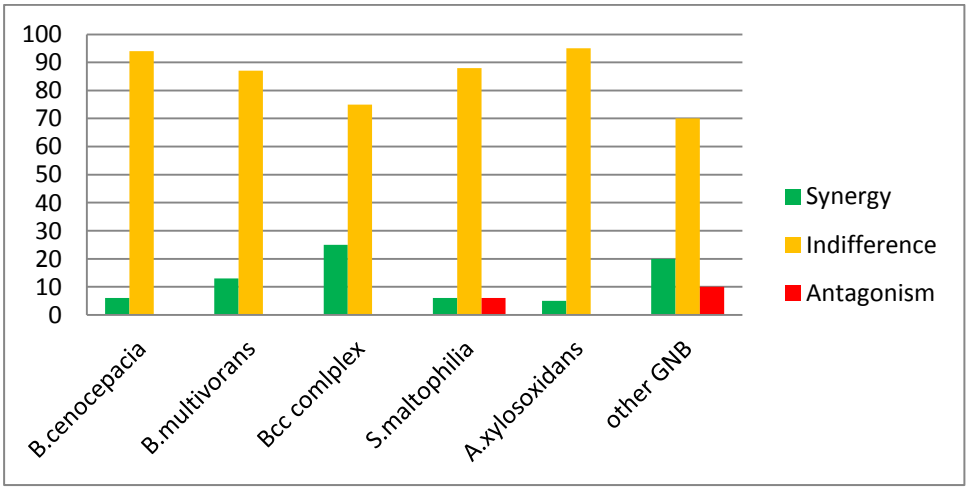

Figure 6.7 Percentage synergy/indifference/antagonism of isolates tested with aztreonam and tigecycline in combination.

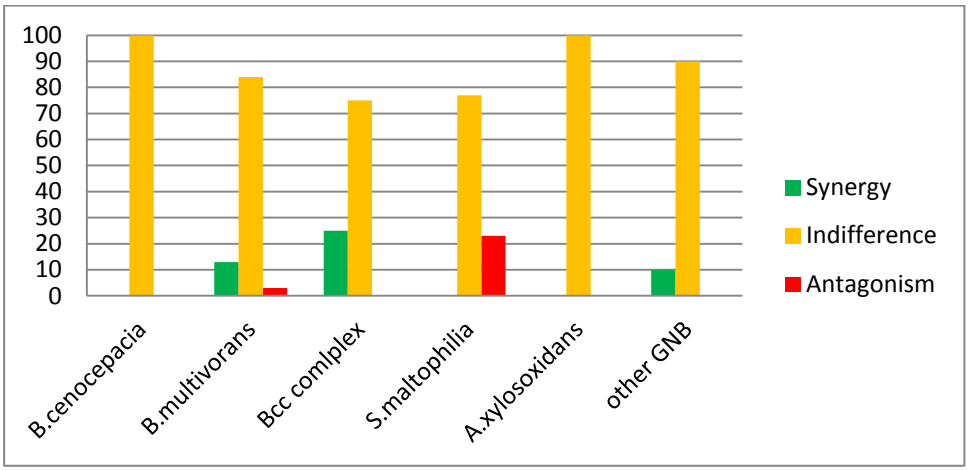

Figure 6.8 Percentage synergy/indifference/antagonism of isolates tested with Tobramycin and tigecycline in combination.

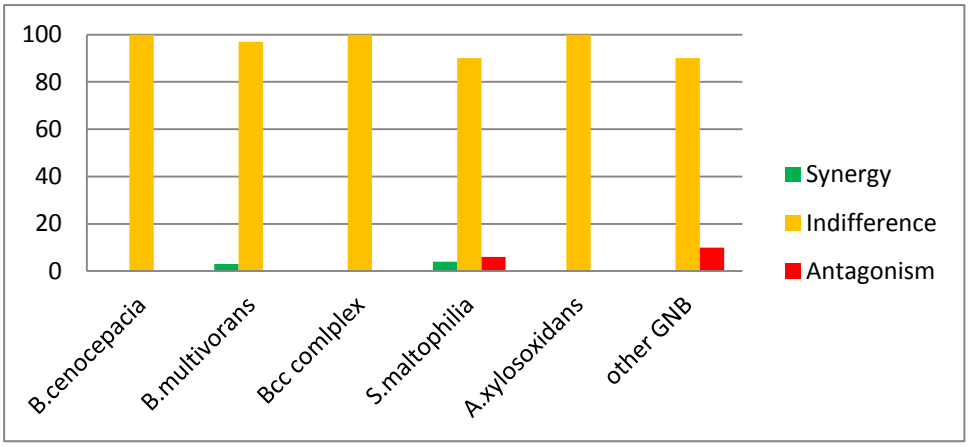

Figure 6.9 Percentage synergy/indifference/antagonism of isolates tested with temocillin and tigecycline in combination.

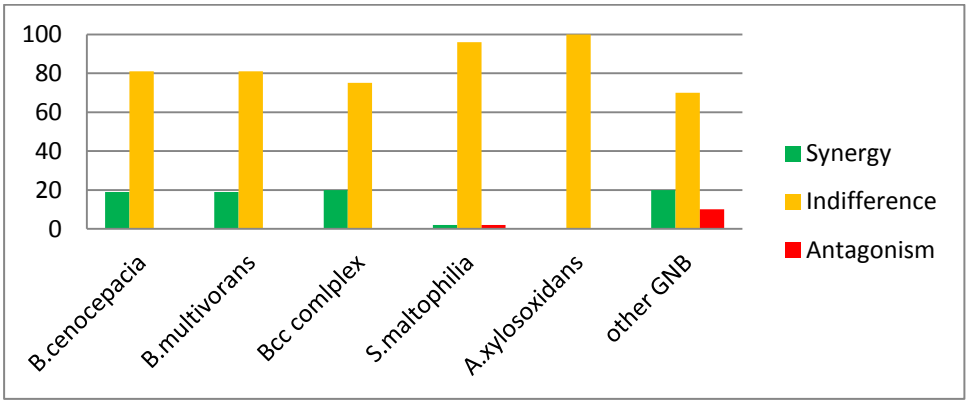

Figure 6.10 Percentage synergy/indifference/antagonism of isolates tested with ceftazidime and tigecycline in combination.

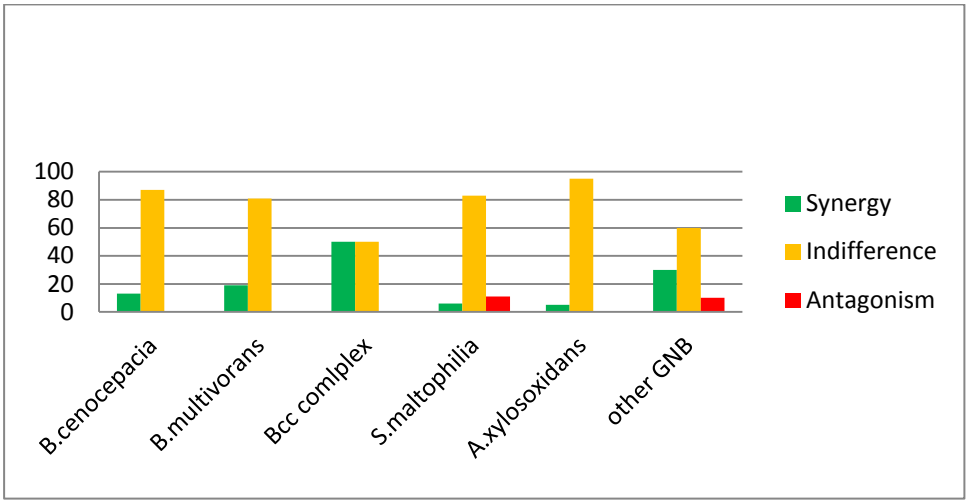

Figure 6.11 Percentage synergy/indifference/antagonism of isolates tested with piperacillin/tazobactam and tigecycline in combination.

6.2.3 *Burkholderia cepacia* complex

Tigecycline demonstrated poor activity against the *Burkholderia cepacia* complex isolates, with only 13% (*B. cenocepacia*), 3% (*B. multivorans*) and 25% (other Bcc spp) being sensitive. A greater proportion of these organisms demonstrated intermediate sensitivity (19, 32, 15% respectively), though how this would translate into the clinical setting is unclear. Figs 6.12 – 6.14 show how tigecycline interacted with each individual species of Bcc tested in this study.

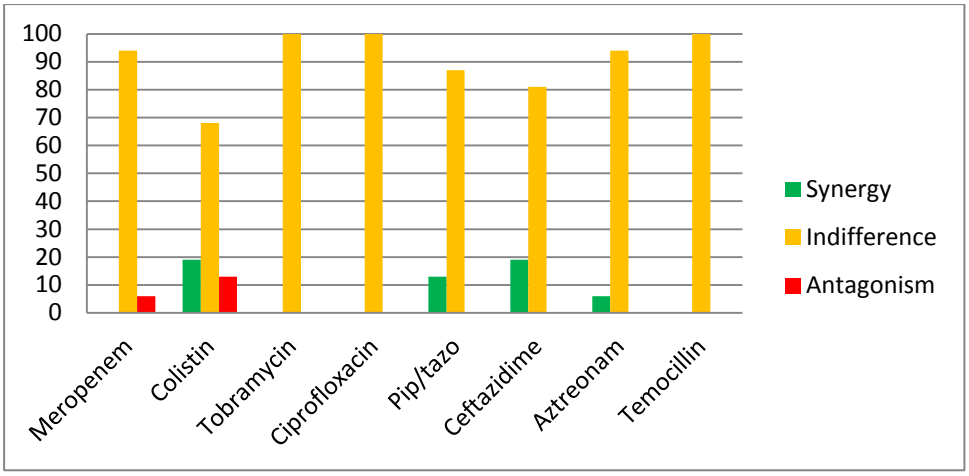

Figure 6.12: Percentage of *B. cenocepacia* demonstrating synergy/antagonism or indifference when in combination with tigecycline

The range of tigecycline MICs was large, indicating significant heterogeneity in our test population and possibly reflecting variability in sensitivity within the Bcc population in general.

In comparison, minocycline - the older analogue of tigecycline, demonstrated greater activity against the same isolates with 94% of *B. cenocepacia*, 91% *B. multivorans* and 75% "other" Bcc spp being sensitive respectively. It also follows, that intermediate resistance/sensitivity was much lower than that of tigecycline. Both the range of minocycline when compared to tigecycline, and the MIC50 and 90 were superior, with minocycline being less variable within our test populations than tigecycline – possibly indicating greater reliability.

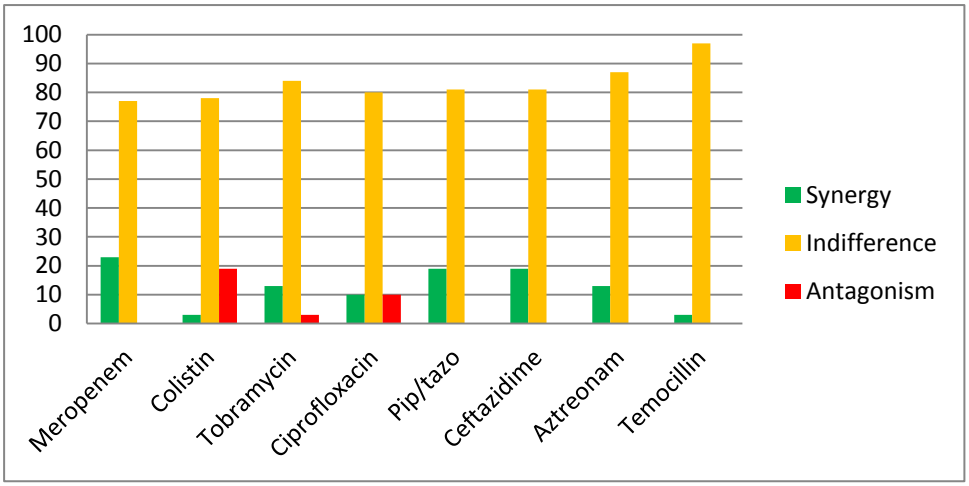

Figure 6.13: Percentage of *B. multivorans* demonstrating synergy/antagonism or indifference when in combination with tigecycline

Colistin showed minimum activity against the *Burkholderia* species with only 3% of *B.multivorans* isolates being sensitive. Whilst Colistin was relatively inactive against the *Burkholderia* isolates tested, synergistic activity was noted. In Figures 6.8-6.10 above, it can be seen that both synergy and antagonism occurred across the *Burkholderia* species groups tested. Of note, is the fact that occurrences of antagonism in this combination was almost equal in proportion to the occurrence of synergy.

Between 50 and 70% of isolates demonstrated indifference leaving rather a confused and variable pattern for colistin in combination with tigecycline. The activity of ciprofloxacin was variable against all isolates in this study. 75% of *B. cenocepacia* species were resistant along with 68% of *B. multivorans*. 75% of "other *Burkholderia* spp" were sensitive to ciprofloxacin, but the low numbers in these particular isolates as previously stated, preclude any real comparison of this pattern with other *Burkholderia cepacia* complex species tested here.

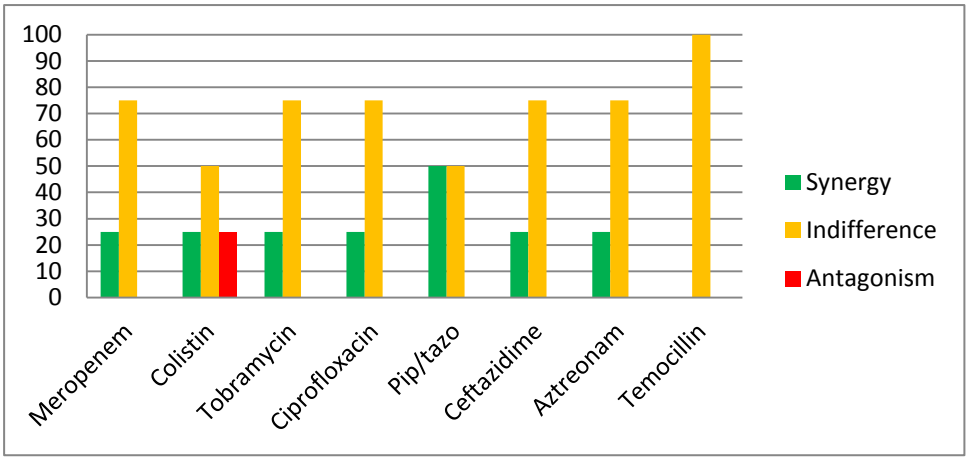

Figure 6.14: Percentage of Bcc "other" strains demonstrating synergy/antagonism or indifference when in combination with tigecycline

In combination with tigecycline, ciprofloxacin was observed to produce the variable results that typified these investigations. Some synergy was observed with *B. multivorans*, and the group of "other" Bcc isolates. Low levels of antagonism were observed, though this seemingly correlated with those isolates that had demonstrated synergy. The vast majority of the isolates tested, however, were indifferent to the combination.

Aztreonam showed variable activity against the *Burkholderia* species. It was most active against *B. multivorans* isolates (19% sensitive, 35% intermediate), with poorer activity against the *B. cenocepacia* isolates (6% sensitive, 19% intermediate). Both sets of isolates showed a large range, further indicating variation in susceptibility throughout the groups.

In combination with tigecycline, aztreonam demonstrated low levels of synergy across the board, with antagonism only occurring at low levels when challenged with *S. maltophilia* and small numbers of the other CF related GNBs (*P. sputorum*). Of particular note was the small number of other Bcc species that demonstrated 25% synergy though the low numbers in this group may have exaggerated this result.

The aminoglycosides tested in this study showed variable and rather low activity across the spectrum of isolates with large ranges in all species. Tobramycin was marginally less effective overall than amikacin, though direct comparison is difficult as amikacin has an

intermediate range that was frequently observed. Using the MIC50 data, it is possible to see that tobramycin had the lower MICs throughout the *Burkholderia* species with 13, 10 and 25% sensitivity to *B. cenocepacia, multivorans* and "others" respectively. In comparison, amikacin was sensitive to only 6, 3 and 25%, but intermediate to 6, 10 and 25% respectively. The lower MICs observed with tobramycin suggest that it is more effective *in-vitro,* though the intermediate range of amikacin may make up for this difference somewhat. Amikacin, in particular, was less effective than tobramycin, particularly with respect to the *Burkholderia* species demonstrating a mean sensitivity of 11% across the isolates. Tobramycin demonstrated marginally more activity against the *Burkholderia* species with an average of 16% sensitivity.

Where tobramycin was combined with tigecycline, low levels of antagonism were recorded – notably against the *B. multivorans* species (3%).

Against *B. cenocepacia, B. multivorans* and "others", temocillin demonstrated 31, 48 and 25% sensitivity respectively. With a low MIC50 and shorter ranges than most of the other antimicrobials tested in this study, it appears that temocillin is a promising antimicrobial when used against the *Burkholderia* isolates *in-vitro*.

Temocillin, demonstrated some activity against the Bcc isolates (31% sensitive to *B.cenocepacia,* 48% *B. multivorans*). Sensitivity against the *Burkholderia* species averaged around 35% compared to 54% sensitivity of the same isolates tested against ceftazidime – another β-lactam antibiotic. This situation was not improved by combining temocillin with tigecycline – with the majority of isolates being indifferent to the combination.

Ceftazidime was relatively effective against all of the species tested in this study. It was particularly effective against *B. multivorans* where 74% susceptibility was observed. With low MIC50 observations, it appears that ceftazidime is a powerful antimicrobial where around 50% susceptibility across the range of species tested was observed. Of the β-lactam

antibiotics tested, ceftazidime, was the only cephalosporin antibiotic tested in this study and was the most effective. It has long been documented to be active against *Pseudomonas* spp and in this study its effectiveness whilst variable against both Bcc and other GNBs from CF patients, was generally good. The *Burkholderia* species averaged around 60% sensitivity across the range. As with many of the antibiotics tested, the testing range was large (1-256) but most of the islolates tested fell within the lower MIC bracket.

In combination with tigecycline, ceftazidime demonstrated good activity with an average of 21% synergy demonstrated across the *Burkholderia* species. No antagonism was noted.

Piperacillin in combination with tazobactam (PTA) was another anti-*pseudomonal* penicillin and was found to be more effective than temocillin *in-vitro*. Against the *Burkholderia* species, the two antibiotics are broadly comparable, with PTA being slightly more active (for example, 58% sensitivity against *B. multivorans* versus 48% sensitivity where temocillin was used).

As with many of the other combinations of antibiotic/isolate, tobramycin ciprofloxacin and meropenem demonstrated variable amounts of synergy. This was only observed in the *B.multivorans* and "other" isolates, though they may have been exaggerated in some cases due to small numbers of "other" isolates. Against the *B. multivorans* isolates, however, meropenem did demonstrate significant levels of synergy that may be useful.

6.2.4 *Stenotrophomonas maltophilia* isolates

Tigecycline showed promising activity against *S. maltophilia* isolates, with 77% sensitivity and 11% intermediate sensitivity/resistance. This was in direct contrast with the results previously described in the Bcc species. When compared with minocycline, however, there remains a significant difference. Minocycline exhibited 96% sensitivity with 2% of isolates tested being intermediate. In addition to this, tigecycline once again showed greater variation though how significant this difference is, where sensitive isolates predominate, is

unclear. The overall results of testing of S.maltophilia against tigecycline in combination with other antimicrobials can be seen below in Fig 6.15.

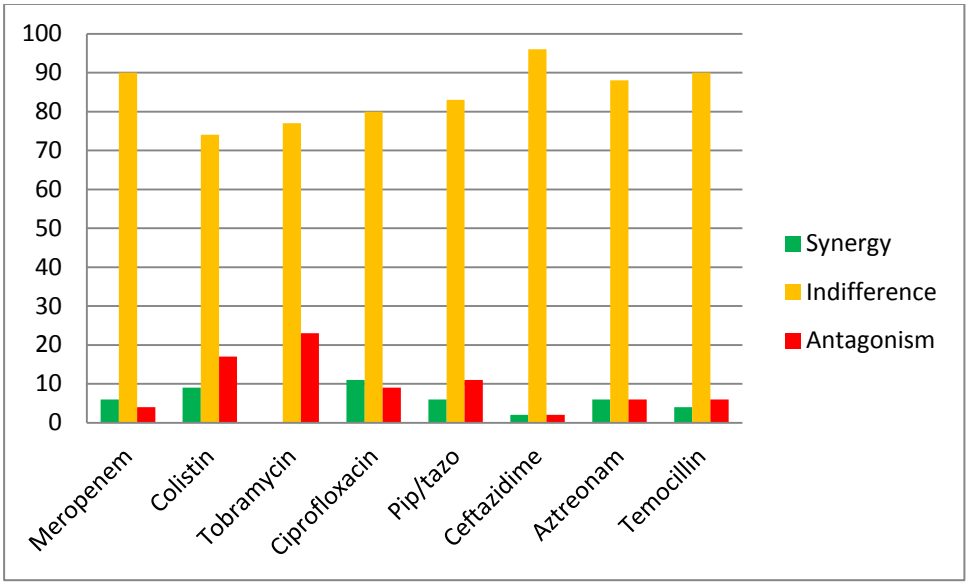

Figure 6.15: Percentage of *S. maltophilia* strains demonstrating synergy/antagonism or indifference when in combination with tigecycline

The most effective antibiotics against *S. maltophilia in-vitro* from this study were minocycline (96% sensitivity) and tigecycline (77% sensitivity). Conversely, the least effective were aztreonam (2% sensitivity) and temocillin (temocillin exhibited no activity against *S. maltophilia*). Whilst temocillin did exhibit some limited synergy, it was outweighed by antagonistic interactions (not noted in other species). The largest single group of isolates in this study comprised of the *S. maltophilia* isolates. It was in this group where the most antagonism was noted – across almost the entire spectrum of antimicrobials that were tested in combination. As with the pattern observed with colistin with other isolate groups, the antagonism in the *S. maltophilia* group appears to match almost exactly the synergy as it occurs. The only antibiotic in this group where antagonism was mutually exclusive, was tobramycin. This was the only group in which these elevated levels of antagonism were noted. In other groups as noted, antagonism was almost entirely absent and promising levels of synergy were observed.

6.2.5 *Achromobacter xylosoxidans*

The activity of tigecycline against this group of organisms was promising, with 85% of isolates being sensitive to this antibiotic. Unlike other groups of organisms, this was comparable to minocycline where 80% of isolates were sensitive. The main difference between the two antibiotics was in the intermediate range where tigecycline showed 10% and mincycline 20% respectively. Only 5% of isolates were resistant to tigecycline whereas none were resistant to minocycline. The results of testing can be seen in Fig 6.16 where A.xylosoxidans was tested against tigecycline in combination with other antimicrobials.

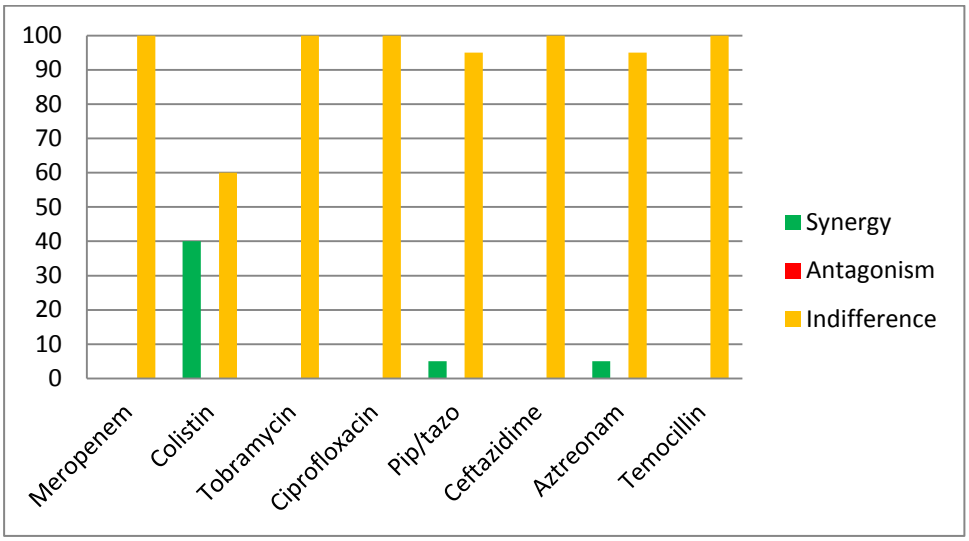

Figure 6.16: Percentage of *A. xylosoxidans* demonstrating synergy/antagonism or indifference when in combination with tigecycline

Tigecycline, minocycline and piperacillin/tazobactam were the most successful antibiotics in-vitro against this group of organisms, with temocillin and aztreonam being virtually inactive. Most of the other antibiotics tested showed significantly reduced activity.

Unfortunately, despite observing synergy in other species tested in this study, very little synergy was observed in the *A .xylosoxidans* group. Previous patterns have indicated that synergy occurs where organisms would otherwise have been resistant. This does not appear to have been the case here.

The only synergy observed within this group occurred with colistin (40%), aztreonam (5%) and piperacillin/tazobactam (5%). The only group of isolates where no antagonism was observed was the *A. xylosoxidans* group. At 40% synergy, the significance of colistin cannot be easily overlooked. Unfortunately, though the lack of antagonism was encouraging, the levels of synergy with other combinations were either completely absent, or very low and it would appear that only colistin in this group generated reliable and significant levels.

6.2.6 Other Gram negative bacilli (GNB) clinical strains

The group of "other GNB clinical stains" was a rather mixed group and was included in this study largely for illustrative purposes. It was recognised that insufficient numbers from which to draw firm conclusions, existed – but that the study may benefit from this additional information in the wider context of CF colonisation/infection.

Table 6.3: Summary of "Other" GNB clinical trains and their response to each antibiotic tested.

	Meropenem	Colstin	Tobramycin	Ciprofloxacin	Pip/Tazo	Ceftazidime	Aztreonam	Temocillin
Shewanella putrefaciens								
Shewanella putrefaciens								
Chryseomonas meningosepticum								
Delftia acidivorans								
Pandoraea sputorum								
Pandoraea apista								
Pseudomonas stutzeri								
Pseudomonas stutzeri								
Delftia acidivorans								
Chryseomonas indologenes								

Synergy	
Indifference	
Antagonism	

Of particular note in this group were the *Pandorea* isolates – *apista* and *sputorum*. These produced almost completely polarised result in that the *P. apista* group demonstrated marked synergy when tested against combinations. *P. sputorum*, however, demonstrated a concerning amount of antagonism. Similarly discrepant results were observed with the *Pseudomonas stuzeri* isolates tested – with one isolate demonstrating synergy almost across the entire range of antimicrobials and the other showing complete indifference. A summary of these results can be seen in Table 6.3

6.2.7 Additive effects.

The decision was made in this study to describe non-synergistic and non-antagonistic interactions as "indifferent". It should be noted, however, that a 4th option does exist but that it may be viewed with some subjectivity (Balke *et al*, 2006). It was also noted that a number of authors do not use the term in their research findings – presumably for the same reasons (Pankey & Ashcraft, 2005; Smith *et al*, 2006; Aaron, 2007). Where the term is used, it is acknowledged that it may be viewed differently by different authors (Saiman, 2007). For completeness, it was decided to include this data as an addendum so that it may be used as deemed appropriate by the reader and we draw attention to the discussion where further mention of this data is made.

Table 6.4: Additive strains as a percentage of the total percentage of indifferent strains.

	Meropenem		Colistin		Tobramycin		Ciprofloxacin		Pip/tazo		Ceftazidime		Aztreonam		Temocillin	
Species Group	Indifferent	Additive	Indifferent	Additive	Indifferent	Additive	Indifferent	Additive	Indifferent	Additive	Indifferent	Additive	Indifferent	Additive	Indifferent	Additive
B.cenocepacia	94	6	68	19	100	6	100	6	87	29	81	16	94	6	100	6
B.multivorans	87	22	78	0	84	12	80	0	81	16	81	32	87	15	97	33
"other *Bcc spp* "	75	33	50	0	75	0	75	0	50	0	75	0	75	0	100	25
S.maltophilia	90	0.6	74	0.4	77	0.4	80	0.6	83	0	96	0.6	88	1	90	14
A.xylosoxidans	100	0.5	60	0	100	0.5	100	0.5	95	0.1	100	0.5	95	0	100	2
"other GNBs"	90	11	70	0	90	0	70	0	60	0	70	14	70	0	90	0

Table 6.5: The minimum inhibitory concentrations (mg/L) of eleven tested antibiotics for each species/genus group. Proportion susceptible (S), intermediate (I) or resistant (R) in relation to defined breakpoints.

		TGC	MIN	AMI	TOB	AZT	CAZ	MER	PTA	TEM	COL	CIP
Burkholderia cenocepacia (16)	Range	1-64	0.25-8	4-256	4-256	1-256	1-256	0.5-32	0.5-256	4-64	32-256	0.5-32
	MIC_{50}	32	2	256	32	256	4	2	32	32	256	32
	MIC_{90}	64	4	256	64	256	64	32	256	64	256	32
	%S	13	94	6	13	6	56	56	38	31	0	6
	%I	19	6	6	0	19	0	25	0	0	0	19
	%R	68	0	88	87	75	44	19	62	69	100	75
Burkholderia multivorans (31)	Range	1-256	0.5-16	4-256	0.25-1024	1-256	0.5-256	0.5-32	0.12-256	2-1024	0.25-256	0.25-32
	MIC_{50}	4	1	256	32	4	2	2	2	8	256	2
	MIC_{90}	64	4	256	512	256	64	32	256	32	256	32
	%S	3	91	3	10	19	74	48	58	48	3	13
	%I	32	6	10	0	35	0	19	0	0	0	19
	%R	65	3	87	90	46	26	33	42	52	97	68
Other *Burkholderia cepacia* complex species (4)	Range	1-16	0.25-16	4-256	1-64	1-256	1-256	1-32	0.016-256	0.5-64	256	0.125-4
	MIC_{50}	2	0.25	16	8	2	2	1	8	64	256	0.25
	MIC_{90}	12	2	256	64	64	4	32	256	64	256	4
	%S	25	75	25	25	25	50	75	60	25	0	75
	%I	50	0	25	0	25	0	0	0	0	0	0
	%R	25	25	50	75	50	50	25	40	75	100	25
Achromobacter xylosoxidans (20)	Range	0.12-8	0.25-8	0.5-256	0.25-1024	32-256	2-256	0.06-32	0.5-256	64-1024	0.12-256	0.03-32
	MIC_{50}	0.5	2	256	8	256	8	0.125	1	1024	4	4
	MIC_{90}	2	8	256	1024	256	256	32	64	1024	256	32
	%S	85	80	10	15	0	60	65	85	0	40	10
	%I	10	20	0	0	0	0	10	0	0	0	15
	%R	5	0	90	85	100	40	25	15	100	60	75
Stenotrophomonas maltophilia (47)	Range	0.12-4	0.12-64	4-256	0.12-1024	1-256	0.25-256	0.25-32	4-256	32-1024	0.06-256	0.25-32
	MIC_{50}	1	0.5	256	32	256	16	32	256	512	2	4
	MIC_{90}	4	2	256	1024	256	256	32	256	1024	256	32
	%S	77	96	9	26	2	47	21	26	0	51	13
	%I	11	2	15	0	9	0	2	0	0	0	9
	%R	12	2	76	74	89	53	77	74	100	49	78
Other CF-associated Gram negative bacteria^ (10)	Range	0.12-16	0.125-16	1-256	0.12-1024	1-256	0.12-256	0.06-32	0.01-256	2-1024	0.06-256	0.01-16
	MIC_{50}	0.5	0.5	256	32	256	8	32	8	1024	256	0.25
	MIC_{90}	2	2	256	1024	256	256	32	256	1024	256	8
	%S	80	100	30	20	10	60	40	60	30	50	60
	%I	10	0	10	0	20	0	0	0	0	0	20
	%R	10	0	60	80	70	40	60	40	70	50	10

TGC: tigecycline; MIN: minocycline; AMI: amikacin; TOB: tobramycin; AZT: aztreonam; CAZ; ceftazidime; MER: meropenem; PTA: piperacillin-tazobactam; TEM: temocillin; COL: colistin; CIP: ciprofloxacin.
^ *Delftia acidovorans* (2), *Pseudomonas stutzeri* (2), *Shewanella putrefaciens* (2), *Chryseomonas indologenes, Chryseomonas meningosepticum, Pandoraea sputorum, Pandorea apista.*

Table 6.6:. Tigecycline in combination with other antimicrobial agents: percentage of isolates demonstrating synergy (SYN), antagonism (ANT), or indifference (IND) for each species/genus group.

		TOB	AZT	CAZ	MER	PTA	TEM	COL	CIP
Burkholderia cenocepacia (16)	%SYN	0	6	19	0	13	0	19	0
	%ANT	0	0	0	6	0	0	13	0
	%IND	100	94	81	94	87	100	68	100
Burkholderia multivorans (31)	%SYN	13	13	19	23	19	3	3	10
	%ANT	3	0	0	0	0	0	19	10
	%IND	84	87	81	77	81	97	78	80
Other *Burkholderia cepacia* complex species (4)	%SYN	25	25	25	25	50	0	25	25
	%ANT	0	0	0	0	0	0	25	0
	%IND	75	75	75	75	50	100	50	75
Achromobacter xylosoxidans (20)	%SYN	0	5	0	0	5	0	40	0
	%ANT	0	0	0	0	0	0	0	0
	%IND	100	95	100	100	95	100	60	100
Stenotrophomonas maltophilia (47)	%SYN	0	6	2	6	6	4	9	11
	%ANT	23	6	2	4	11	6	17	9
	%IND	77	88	96	90	83	90	74	80
Other CF-associated Gram negative bacteria^ (10)	%SYN	10	20	20	10	30	0	20	20
	%ANT	0	10	10	0	10	10	10	10
	%IND	90	70	70	90	60	90	70	70

TOB: tobramycin; AZT: aztreonam; CAZ; ceftazidime; MER: meropenem; PTA: piperacillin-tazobactam; TEM: temocillin; COL: colistin; CIP: ciprofloxacin.
^ *Delftia acidovorans* (2), *Pseudomonas stutzeri* (2), *Shewanella putrefaciens* (2), *Chryseomonas indologenes, Chryseomonas meningosepticum, Pandoraea apista*

Chapter 7

7.0 Discussion

7.1 PFGE and epidemiology

One of the aims of this study was to ascertain the epidemiological spread of the isolates that have been obtained in the Leeds Cystic Fibrosis Centre over the last ten years. This was primarily to enable the exclusion of duplicate isolates for MIC/synergy testing, though the PFGE work does tentatively draw some epidemiological conclusions. The long-term colonisation of patients with Bcc and other organisms has serious implications for infection control within the Leeds CF unit, but also within the CF community (Govan *et al*, 2007). As has been mentioned previously, the stigma attached to carriage of Bcc organisms is such that the psychological effect upon patients can be pronounced (McDowell *et al*, 2004).

Notwithstanding potential duplicate isolates and the small number that were unrecoverable from storage, *B. multivorans* was the predominant organism in the LTHT historical collection. It is difficult with small numbers and a historical collection to draw firm conclusions, though as stated in the introduction, this may indicate that colonisation/infection of the patients in this study was sporadic and may have originated from environmental sources rather than patient to patient spread (McDowel *et al*, 2004). There have been a number of studies that have found centre specific predominance of Bcc strains that differ from environmental strains. The findings of this study, however, do not appear to support this work and it has been noted that in some units, there can be a large spread of genetically differing species with not all isolates necessarily carrying specific markers of transmissibility (Mahenthiralingam *et al*, 1996, Mahenthiralingam *et al*, 2002).

Some historical work has found similar results where a small number of isolates did not cluster when examined (Steinbach *et al*, 1994), though in most studies, patient to patient transmission within CF units has been found (Speert *et al*, 2002). This indicates that, whilst patient to patient spread is important, it does not necessarily follow that all patients within a CF unit will harbour the same strains of Bcc, and that this may be dependent upon the origin of the strains and the predominance of specific types – such as *B. multivorans*. It has also been suggested that despite segregation in CF units, patients will still be sporadically colonised by Bcc organisms from the environments in which they live (McDowell *et al*, 2004). The findings in this study may also indicate that the infection control policies of the LTHT have been relatively successful over the last ten years. Had this not been the case, this study may have found more pronounced clustering of isolates as similar strains are detected in multiple patients. Furthermore, given the findings of previous work and the patient to patient transmission data presented; it may seem reasonable to tentatively postulate that patients within the LTHT CF unit have likely acquired their strains from the environment and that nosocomial spread has been minimal (Speert *et al*, 2002, Mahanthiralingam *et al*, 2002, McDowel *et al*, 2004). This, therefore, would represent promising data in terms of the practices of the LTHT CF unit.

As the major route of transmission for organisms such as *S. maltophilia* and *A. xylosoxidans* may be environmental, it is difficult to accurately quantify the effect that infection control measures and isolation may have had with regard to these organisms. The evidence from PFGE typing, however, is that the isolates in the LTHT CF unit appear to be, for the most part, unique – suggesting that acquisition was either from multiple external/environmental sources, or that the LTHT CF unit infection control measures may have had some beneficial outcomes where nosocomial spread was possible (Denton *et al*, 1998). Our work on these organisms has shown some agreement with previous studies in this area – suggesting that

our collection of isolates is not unique in their diverse nature. Large studies by Valdezate *et al* (2004) and Denton *et al* (1998) demonstrated that in a single, large CF unit, there was very little relationship between isolates. This has been matched by our findings, and confirms to some degree the hypothesis that these organisms are also acquired from the environment and not necessarily from other patients.

The continuous monitoring of microbiological colonisation of patients within the LTHT has clearly paid dividends in terms of proactively seeking to identify those patients who are, unfortunately, harbouring pathogenic species such as Bcc and then putting into place protective measures to prevent the further spread of these organisms.

Epidemiology aside, the main reason for testing isolates by PFGE, was to eliminate duplicate isolates. The benefit of this is to achieve a data set that is representative not only of the LTHT CF unit, but that potentially gives an indication of national trends. Had we included large numbers of repeat isolates or duplicates that have come from multiple patients (as part of an outbreak, or nosocomial spread within the CF unit), then the data would have been skewed to benefit/disadvantage individual antibiotics. Our collection, therefore, gives a cross sectional, representative sample of organisms isolated from CF patients in the LTHT CF unit without prejudice to age, gender or other demographics. Where similarities were observed, we investigated further to ensure that the isolates were phenotypically different – as can be seen in the examples provided in the next section. No complete and convincing duplicates were found in this study.

7.1.1 Heterogeneity/variability of strains and testing

MIC testing gave an overall picture of the performance of each individual antimicrobial against each group of isolates, thereby providing a baseline upon which to extrapolate the results of synergy testing. This information was also useful as a likely indicator of clinical

efficacy amongst those patients regularly seen in the LTHT CF unit. The clinical relevance of this data is discussed later, but a number of patterns emerged that may potentially inform the clinician about the likely outcomes of antimicrobial chemotherapy. Combining the MIC data with that gained from the PFGE investigations gave an interesting picture of the diversity of the organisms found in the LTHT CF unit and may shed some further light not only on the likely epidemiology described earlier, but also on future patterns that may develop as a result of the relative genetic promiscuity of these organisms, and the testing issues that may arise as a result of this. It is these issues of heterogeneity that are discussed here.

Since differences in susceptibility between organisms are quite normal – especially in CF patients, a small number of "closely" related strains are illustrated here to demonstrate the variability that can be observed when conducting MIC testing (Doring *et al*, 2000; Fowraker *et al*, 2010). One example of the polymorphisms exhibited by CF isolates is demonstrated by the three ET12 epidemic strains of *B. cenocepacia* that were investigated in this study. Not surprisingly, there was some clustering of these organisms, though by Tenover's criteria (1995), it would appear that these are likely to be different strains. We know from past work, however, that the ET12 lineage, whilst widespread in hospitals, is part of a small group of strains that have rarely been seen in other environments (Holden *et al*, 2009). Whilst these strains may genuinely differ (indicating that they are unique in origin), it may be that other factors such as insertions and other random genetic events may have had an influence. The genomes of a number of ET12 isolates have been sequenced in recent years, and the variable expression of virulence (including differences in antimicrobial susceptibility) investigated. It was found that the ET12 strains differed from others by up to 21% indicating that genetic transfer plays a major part in their virulence and adaptability to the host environment. It is also likely that this plasticity may have an influence where the

strains are typed by PFGE (Holden *et al*, 2009). We found that our three isolates differed in their susceptibility to tobramycin (three differences), ceftazidime (one difference) and temocillin (one difference). These differences are difficult to explain without sequencing the genomes of the respective isolates. However, previous work by Holden *et al* (2009) suggests that ET12 strains possess multiple resistance genes encoding efflux pumps, siderophores (that, when activated help to protect against the reactive oxygen radicals encountered during treatment) and β-lactamases. In their study, it was noted that the expression of these genes was variable, and could be influenced by the medium within which the bacteria grow (in their case, CF sputum). Up to eight drug efflux pumps were detected by Holden *et al* (2009), and it may be reasonable to hypothesise that some of these may have been present in some of the isolates investigated in this study. Since both ceftazidime and temocillin are β-lactam antibiotics, it is likely that they, too, may have been susceptible to upregulated β-lactamase and penicillin binding protein expression. Again, it is difficult without sequencing, to state unequivocally which mechanisms may be responsible, but a number have been noted to be present – including a highly conserved metallo-β lactamase of uncertain significance (it is not known at present whether this is a functionally active domain). The variable expression of these resistance markers means that there are a large number of combinations possible from even small numbers of isolates – leading to significant heterogeneity in both scientific end points (such as MICs), and clinical outcomes. This effect upon the heterogeneity of clinical outcomes has been demonstrated in a number of studies and appears to be a likely confounder in any treatment prediction that results from this type of testing (Speert *et al*, 2002; Fowraker *et al*, 2010).

Another aspect that fell outside the remit of this study, relates to the current and past treatment of patients. It has been demonstrated that some antibiotic resistance mechanisms are potentially treatment induced (for example the *ceo* efflux pump – which is activated

when the patient is treated with chloramphenicol, trimethoprim or ciprofloxacin). Where inducible effects occur – relating to antimicrobial therapy, growth environment and genetic events, these will increase the heterogeneity of the test population.

Finally, the additional effect of heterogeneous populations is something with which any CF microbiologist has to grapple. As mentioned previously, it is well reported that testing can demonstrate, even from single colonies, multiple morphotypes that will differ in their susceptibility. Up to three morphotypes were found in a number of studies (Aaron *et al* 2007; Fowraker *et al*, 2010) when testing *P. aeruginosa*, and this effect is common throughout CF isolates (Denton & Kerr, 1998). Test populations, therefore, may produce variable results. It is possible that duplicate isolates may be tested resulting in apparently completely different MIC data. As stated previously, this variation is not limited to the Bcc species and *P. aeruginosa*. Small colony variants (SCV) of *S. maltophilia* have been noted, and these are difficult to culture using standard methods (Anderson *et al*, 2007). At present, it is difficult to quantify the effect that they may have on testing due to their unique growth characteristics. We cannot, therefore, rule out the possibility that these organisms are present in our, or any test population or their influence upon the results. These SCVs have been found in numerous pathogenic organisms, and whilst there is currently no data relating to the other species tested in this study (Bcc spp/*A. xylosoxidans*), this may be an area for further investigation and may additionally confound susceptibility testing.

The historical lineage of many CF related organisms, is environmental. *S. maltophilia*, for example, has been found in many habitats including plants, water, soil sediments and organic residues. It is thought that this diversity of habitats may be one of the factors in the metabolic diversity of the organism – thereby assisting the heterogeneity of both testing, and outcome with these organisms. Other differences in susceptibility – both to single and multiple antibiotic combinations may be due to differences in resistance mechanisms both

acquired and innate. One example of this is the multi-drug efflux pump "*sme*" – this is a pump that in combination with its outer membrane protein (OMP), energy dependent transporter and membrane fusion protein, is capable of pumping antibiotics from the bacterium *S. maltophilia*. Notably, however, this pump is not associated with the multi-resistance phenotype. Conversely, other isolates that have been studied have been found to have completely different pump mechanisms such as the similarly named "*smeDEF*" pump (Alonso & Martinez, 2000) – which is associated with multi-resistant phenotypes. When sequence analysis was carried out on these two pumps they were noted to be quite different – indicating that whilst *S. maltophilia* may be widespread within a CF unit, the susceptibilities can vary considerably due to the differing mechanisms of resistance that may be present.

In all of the organisms tested here, there is the issue of biofilm and differential growth curves. Studies have noted that bacteria exhibit differing levels of resistance depending upon their growth stage – with resistance increasing markedly during the exponential growth phase and topping out at the stationary phase during which bacteria are located in the biofilm (Desai *et al*, 1998). As stated later in the discussion of limitations and further work, it is difficult to propose a way of including this data – and indeed detecting the growth phase of the organism concerned and would likely be prohibitive. We did time the application of organisms into growth media as accurately as possible, though it is difficult to know where the organisms originated from in the patient in terms of growth phase or biofilm location, and there may have been some experimental variability that could influence these factors. It should be noted that in a clinical environment, organisms are less likely to be uniform in their growth phases, though this has been controlled as much as practicable within this study.

7.2 MIC/synergy testing

The MIC ranges found with most of the test antibiotics were very wide – except for a limited number of agents such as meropenem. This may be due to a number of possibilities – chiefly the presence of resistant mutant strains among the test population. Additionally, the effect of bacterial heterogeneity as previously discussed cannot be discounted. The larger ranges observed with older and more established antimicrobials are not surprising, as the use of these antimicrobials may have induced resistance over time.

7.2.1 The tetracyclines

Tigecycline was considerably less effective *in-vitro* than its legacy minocycline derivative against the Bcc species, though it was far more effective against *S. maltophilia* and *A.xylosoxidans* isolates. Thirteen percent of *B. cenocepacia* isolates were sensitive to tigecycline compared to 94% sensitivity to minocycline. Similar results were observed when *B. multivorans* was tested. It is difficult to qualify these differences in susceptibility – particularly as the antibiotics are similar in overall structure. One can only postulate that there may be subtle differences in the uptake of tigecycline by the Bcc species and that this is sufficient to make a significant difference. Other resistance mechanisms such as efflux pumps have yet to be characterised, though it is likely that these are present as they have been observed in other similar species and are noted to have homologues.

It should be noted that the data relating to both minocycline and tigecycline can be skewed somewhat as BSAC/CLSI guidelines are updated and amended. In the latest version that we used for this study (2010), an intermediate range was given. There is some evidence to suggest that where organisms are classed as resistant to antimicrobials *in-vitro*, then organisms *in-vivo* may still respond – possibly due to host factors working in combination

with antimicrobial therapy (Aaron *et al*, 2006). It may be that these intermediate organisms may be susceptible in some of these cases, though this falls outside the remit of this study. Intermediate sensitivity/resistance is largely subjective and depends upon the set of guidelines used. It should be noted that where Bcc appeared to be resistant to tigecycline (compared with minocycline), 19% of *B. cenocepacia* and 32% *B. multivorans* had intermediate sensitivity/resistance to tigecycline respectively. Whilst the difference between sensitivity and resistance to tigecycline and minocycline is substantial, this may bridge that gap. It does, however, suggest that minocycline would be the more useful antimicrobial agent for use in patients colonised with Bcc organisms.

More promisingly, tigecycline was comparable to minocycline against all of the other species tested in this study in that around 80% sensitivity was observed, though whether it would be a better choice clinically is another issue that would have to be investigated further. Tetracycline efflux pumps have been noted in past work, but it is stated in a number of articles that these are not effective against tigecycline – indeed it is noted that this is one of the selling points of this antimicrobial (Greer, 2006). Likewise, tigecycline is noted to be "resistant" to ribosomal protection mechanisms. It is difficult, therefore, to predict accurately why tigecycline is both relatively inactive against the whole spectrum of Bcc species, but also why it is less active than its legacy derivative; minocycline. A number of possibilities exist but are as yet unproven as work has not yet been carried out. Differences in the uptake of tigecycline when compared with minocycline may mean that the antibiotic is reduced in its effectiveness due to a lack of penetration. Additionally, it is possible that there are, as yet, undetected efflux pump mechanisms or changes to the outer membrane proteins that are able to work in combination with this reduced uptake. Interestingly, though we did not test minocycline further, a study by Petersen (2009) found synergy between tigecycline and minocycline, though the mechanisms for this are not understood. Where

synergy occurs with tigecycline, it is possible that the cascading effect of the second antimicrobial may break down or inhibit the appropriate mechanisms, allowing tigecycline to both penetrate and be effective. Assuming, however, that the latter mechanisms are responsible for synergy, this does not fully explain why it is such an ephemeral and hetrogenenous phenomenon. As yet, there is little data relating to tigecycline and minocycline although a study carried out by in the United States in 2000 found that minocycline was active against *S. maltophilia* and Bcc species and our data shows concordance with this work (Kurlandksy & Fader, 2000). Other work has found that doxycycline and the tetracyclines in general are effective *in-vitro* against *S. maltophilia* and *A. xylosoxidans* and our work agreed with these observations (Vartivarian *et al*, 1994; San Gabriel *et al*, 2007). One issue with these antibiotics however, is that they are bacteriostatic and the resulting likelihood of resistance is higher as they are likely to leave small sub-populations uninhibited. Using the tetracyclines in combination with bactericidal antibiotics may solve this problem though it has been suggested that any treatment should be at, or near the highest doses tolerable to mitigate the potential effects of developing resistance (Vartivarian *et al*, 1994).

The Bcc isolates in general showed great variability in both MIC, and synergy/antagonism across the spectrum of isolates and antimicrobials tested. The most successful antibiotics in combination with tigecycline appeared to be ceftazidime, piperacillin/tazobactam and aztreonam – though, there are a number of problems that may prevent these combinations from being used clinically. Ceftazidime has a history of being less effective *in-vivo*, whilst aztreonam simply was not effective alone – and its resulting combination MIC would not necessarily translate to a successful clinical outcome as a result. Piperacillin/tazobactam remains a good performer, though its use in combination with tigecycline would have to be monitored until further data is available.

Whilst a number of antimicrobials were effective against the *S. maltophilia* isolates, combination testing highlighted a number of serious issues. Antagonism was widespread and in most cases outnumbered the instances of synergy. This would suggest that the use of *any* combination should be after rigorous testing in order to avoid clinical failure. The poor synergy observed in these isolates would indicate that combinations that include tigecycline and one other antibiotic may not be effective. Further work may indicate that triple combinations can overcome these issues.

40% of *A. xylosoxidans* isolates were sensitive to colistin – 40% of the isolates were found to be synergistic. Unfortunately the two effects are, for the most part, mutually exclusive. The usefulness of combination therapy using tigecycline must be questioned when treating patients with these organisms. The lack of synergy was disappointing, as many of the antibiotics tested were, in fact, quite efficacious. It does, however, raise the question of whether synergy testing is absolutely necessary against this particular group of organisms, when antibiotics are effective *in-vitro* by themselves. Combination testing may help to further our knowledge of other combinations (not necessarily including tigecycline) and could help to reduce the development of resistance over time.

7.2.2 Tobramycin

The lack of sensitivity of the Bcc species to tobramycin was concerning. Since nebulised tobramycin is in common use with CF patients, it is disappointing to find that 75% of the organisms tested were resistant. This may represent increasing levels of resistance over time resulting from the long term use of nebulisers - a similar situation was observed with all groups of organisms tested. This would represent an area where future expansion of this a study would be useful as it is difficult to make any conclusions in respect of this without further information. To confirm developing resistance, a time linear testing method would

be required. This brings up the issue of non-duplicate isolates. Since it is presumed (after PFGE testing) that all or most of our isolates are possible environmental organisms and that they are unique, then the effects of aggressive antimicrobial therapy may not, in fact, have influenced the susceptibility of these isolates. Further work is warranted. It may also be noted that testing concentrations where disc tests and other methods are used, do not necessarily reflect those that are achieved therapeutically. Aaron *et al* (1999) tested tobramycin at much higher concentrations that would be more representative of those achieved through nebulisation (they cannot be achieved intravenously). It has also been found that some bacteria – notably Bcc species are far more resistant to tobramycin in biofilms when compared to other common CF bacteria such as *P. aeruginosa*. This is true even when the bacteria are harvested and dispersed before culture (Desai *et al*, 1998). It is difficult, therefore, to determine how effective our MIC/synergy testing of these antibiotics would translate where we have determined an antibiotic to have an MIC that would be classed as a resistant organism, and it may indicate that current BSAC and CLSI guidelines are inadequate for specific groups of patients and antibiotic/bacterial combinations. As stated previously, the rather open ended objectives of treatment in CF patients make the view of "sensitive" and "resistant" somewhat subjective. The epidemiological data, suggesting that isolates are environmentally acquired indicates that any resistance mechanisms to aminoglycosides must be either very widespread or chromosomally mediated – such as active efflux pumps as seen in *Pseudomonads*, and the presence of phosphorylating enzymes. Low levels of synergy were observed with the combination of tobramycin and tigecycline. These low levels of synergy may be of some benefit where the patient is empirically treated with tobramycin and then tigecycline is added. However, the low levels of synergy may also indicate that the clinical benefit is, at best tenuous.

Very little synergy with tigecycline was noted except in the case of *B. multivorans* isolates, though this was only in the order of 13%. More concerning, were the higher levels of antagonism that were observed with *S. maltophilia* isolates. Other than colistin which demonstrated the highest overall levels of antagonism, tobramycin demonstrated 23% antagonism against *S. maltophilia* isolates and 3% against *B. multivorans*. It would appear from the data, that tobramycin is unlikely to be of realistic use in combination with tigecycline, or that testing before use would be warranted to prevent adverse reactions and clinical failure.

7.2.3 Colistin

Of all the antibiotics tested, colistin demonstrated the most synergy – particularly against *A.xylosoxidans* isolates where 40% synergy was observed. Indeed, colistin/tigecycline was one of the most synergistic combinations across the whole spectrum of isolates. This presents an interesting issue of how useful this combination is likely to be in the clinical situation. Colistin is available in nebulised form, and has previously been shown to be more effective at the higher concentrations achievable by nebulisation – even when resistance is indicated (Gabriel *et al*, 2004). Tigecycline, however, is not available via this route and would have to be administered parenterally. It remains to be seen how this differential administration would affect the synergistic interactions of the drugs and unfortunately this falls outside the remit of this study. We did not take into account these higher concentrations though, as previously noted, most synergy was detected in isolates that would otherwise be resistant or borderline resistant. It may be possible that we did not detect synergy in low or high MIC organisms at the limits of the spectrum due to limitations of the E-testing method – see later in this chapter for discussion of these issues.

Unfortunately, despite colistin/tigecycline being one of the most successful combinations in terms of observed synergy, the combination also yielded significant antagonism – the most observed in any combinations used in this study. It is difficult to predict why this has occurred as the action of colistin to damage the membrane of the cell in the catastrophic way observed, would potentially allow tigecycline to penetrate better than if it was administered alone. Further work would be needed to ascertain these mechanisms, though it is possible that the two antibiotics simply react under specific circumstances and in the presence of cellular or host factors.

7.2.4 Ciprofloxacin

Ciprofloxacin was variable against the isolates in this study, and was not particularly effective against the Bcc. There is suggestion that this antibiotic may be useful in a nebulised form, though this is not currently available. When tested in combination with tigecycline, ciprofloxacin showed mainly indifference with some low level synergy. In previous work, ciprofloxacin has been found to be less effective against the Bcc than in others. In combination, however, it has been demonstrated to significantly enhance the killing power of combination with meropenem, piperacillin/tazobactam and imipenem. It did not, however, assist other β-lactamase agents (Bonacorsi *et al*, 1999). Ciprofloxacin showed a wide range of MICs against the *B. multivorans* isolates, suggesting the possibility of mutants within the population, though the range was very narrow when tested against *B.cenocepacia* isolates and this has been previously documented in *P. aeruginosa* (Gold *et al*, 1983). It was suggested by Bonacorsi *et al* that this is possible in the Bcc species as the migration of insertion sequences within the chromosome can alter the expression of chromosomal resistance genes (Bonacorsi *et al*, 1999). Work has historically found steadily increasing resistance of *A. xylosoxidans* to ciprofloxacin – and that this has increased with

exposure (isolates identified as "older" were found to be more resistant). This resistance was in accordance with our findings and suggests that ciprofloxacin may not be appropriate for treatment of these organisms (Vartivarian *et al*, 1994). Additionally, there was no synergy noted when ciprofloxacin was used in combination with tigecycline against *A.xylosoxidans*, indicating that the additional killing effect observed with other antimicrobials is not effective in this case.

Ciprofloxacin was variably active against the *S. maltophilia* isolates as previously described (Denton & Kerr, 1998) and some synergy was observed. As with colistin and tobramycin, however, the occurrences of synergy appear to be matched to an extent by the appearance of antagonism in other isolates. Given that the killing effect of ciprofloxacin in combination has not been observed with any of the organisms used in this study, and that antagonism is common, it would seem prudent to not use this combination in the clinical environment – at least until isolates have been tested and antagonism is ruled out.

7.2.5 The β-lactam/carbapenem group: ceftazidime, piperacillin/tazobactam, temocillin and meropenem

Of the β-lactam antibiotics tested, ceftazidime was the most active against all of the organisms tested, with an average of around 50% susceptibility. It is well documented that the organisms tested in this study possess numerous β-lactamase enzymes and other resistance mechanisms, and it has also been shown in past studies that even where results are promising *in-vitro*, this does not necessarily translate to the clinical situation (Govan & Deretic, 1996). The risk of using ceftazidime *in-vivo* is that the likelihood of cephalosporinases, β-lactamases, drug-efflux and reduced porin channel access being possessed by the target organisms tested here, is high. However, this may be addressed to some extent by using ceftazidime in combination. It remains to be seen what the optimal

combination for this antimicrobial would be, but with good activity against around 50% of all isolates tested and with a relatively low MIC 50, it would, *in-vitro*, indicate that the combination of ceftazidime and tigecycline could show some promise. Except for *A.xylosoxidans* where no synergy was observed, and *S. maltophilia* where only 2% synergy was observed, the ceftazidime-tigecycline combination averaged around 20% synergy. If this combination was further combined with a β-lactamase inhibitor, one may imagine that the combination may be more potent. The caveat regarding inhibitors, however, must be that *S. maltophilia* has been demonstrated to possess both L1 metallo and L2 serine-β-lactamases. These are able to overcome both β-lactam antibiotic, and the β-lactamase inhibitor combinations. The fact that synergy was observed in these combinations (minus the inhibitor) suggests that the combination of tigecycline-ceftazidime may be able to somehow bypass these mechanisms, though as can be seen by the low levels of synergy, this may be purely sporadic and incidental. Where we did test an antibiotic-antibiotic-inhibitor combination (tigecycline-piperacillin-tazobactam) we found very little difference in performance. It is unfortunate, that with such good and promising *in-vitro* results, previous studies and clinical work have found that despite this, there is a lack of clinical efficacy of this antibiotic (Gold *et al*, 1983; Desai *et al*, 1998), though this may be related to the bacterial presence of a biofilm. These biofilms have been found to significantly raise the MIC of both piperacillin/tazobactam and meropenem in the Bcc species and it is conceivable that this may have some influence upon the efficacy of ceftazidime and ultimately, the potential use of ceftazidime in combination with tigecycline (Caraher *et al*, 2007). Further work to ascertain the efficacy of the combination of ceftazidime and tigecycline may be indicated for this promising combination. Our results match those of earlier studies, where the most effective β-lactam antibiotics against the Bcc organisms were found to be ceftazidime, meropenem and temocillin (Bonacorsi *et al*, 1999).

Comparatively, the antibiotic/inhibitor combination of piperacillin-tazobactam-tigecycline does not appear to have fared much better than the tigecycline-ceftazidime combination. Piperacillin-tazobactam-tigecycline gave very similar results to ceftazidime and its effectiveness when not in combination was broadly similar – though slightly less effective against *S. maltophilia* suggesting that the β-lactamases in this case may not have been bypassed/inhibited. It may be possible to argue on this basis, that the inhibitor may play no part in synergistic interactions though further study would have to be carried out to ascertain this. More concerning, was the 11% antagonism seen when the combination was used against *S. maltophilia*. Historically, antibiotics such as aztreonam have been tested in combination with an inhibitor against *A. xylosoxidans* (Vartivarian *et al*, 1994). It was noted that only a 2:1 ratio of inhibitor-antibiotic would be effective *in-vitro* – the issue being that serum levels of the inhibitor drop rapidly allowing the antibiotic to be inactivated. This may limit such options and it is unclear how this would be useful clinically.

There is some evidence to suggest that antibiotics such as temocillin may still bring about a positive outcome, despite appearing to be resistant *in-vitro*. A study was carried out in 1992 by Taylor *et al* where clinical improvement was observed in patients that were colonised with isolates previously established as being resistant. This suggests that higher therapeutic doses may be beneficial. However, in our study, we found that temocillin was not effective *in-vitro* against *A. xyolosoxidans* or *S. maltophilia* isolates. Activity against the Bcc species in general was more promising, though only in the order of around 30% sensitivity. This loosely agrees with previous work by Lekkas *et al* (2006) though their work measured outcomes where temocillin was used. Unfortunately they were unable to categorically state that temocillin was individually beneficial, as their patients also received (as is standard practice) an aminoglycoside.

Unfortunately, synergy was rarely observed when tigecycline was tested in combination with temocillin. Had synergy been common, it may have been possible that this would further enhance clinical outcomes where resistance has been detected. With the combination of higher dosing, and a synergistic effect, the resistance mechanisms could potentially have been overwhelmed. Unfortunately, in the case of temocillin, it was more common – though by no means predominant, to observe antagonism when the combination was tested against *S.maltophilia* and other multi resistant GNBs. It would seem prudent therefore, in this case, to avoid using temocillin in combination with tigecycline. Curiously, our results do not match some previous work carried out in 1999 by Bonacorsi *et al*, where temocillin was found to be the most active β-lactam agent. It is difficult to explain this seemingly different picture of resistance, other than to suggest that the populations tested differ geographically (their study was carried out in France), and may therefore, have acquired differing resistance factors.

Meropenem as a β-lactamase stable carbapenem had mixed results demonstrating around 50% sensitivity to all of the organisms tested except, as expected, for *S. maltophilia*. In combination with tigecycline, meropenem demonstrated 23% synergy (*B. multivorans*), though the pattern for all other organisms was predominantly indifference. These data match previous work, and we note that in other studies, the MIC range was relatively narrow. It was suggested previously (in 1999 by Bonacorsi *et al*) that this may have been due to the recent introduction of the drug, though the situation some 11 years later appears to be similar and this may be promising for the long term use of meropenem against the Bcc and other species tested here (excepting *S. maltophilia*). Meropenem demonstrated good levels of synergy against the *B. multivorans* (23%) though no synergy was observed with *B.cenocepacia* – there being only antagonistic activity (6%) other than indifference. Once again, and following the pattern of otherwise resistant antibiotics, there was some low level

synergy observed when the combination of meropenem/tigecycline was tested against *S.maltophilia*. Given the overall lack of activity of meropenem against *S.maltophilia*, however, it seems likely that this combination would not be clinically useful. Likewise, any decision to treat with the combination against *Burkholderia* species would have to be carefully considered due to the antagonism noted with *B. cenocepacia*. We cannot say from this study whether this is species specific, or merely an anomaly of testing with these two different sets of isolates.

7.2.6 Aztreonam

Aztreonam is noted to have variable activity against the Bcc species and little activity against *S. maltophilia* and *A. xylosoxidans* - including in combination with β-lactamase inhibitors (Vartivarian *et al*, 1994; Denton 2008). Our findings in this study appear to agree with these observations, though the susceptibilities of the Bcc species tested here were somewhat disappointing A study in 2009 by Poirel *et al*, found that *B. multivorans* harbours a chromosomally active Class A β-lactamase (PenB). This is induced via a *LysR*-type transcriptional regulator similar to that found in *Proteus vulgaris* and a number of other organisms, though the trigger is unclear. The narrow spectrum of activity of this enzyme includes aztreonam. Once again, whilst it is difficult without additional data to hypothesise a reason for differential expression of such resistance mechanisms or, in fact, their presence in the organisms tested here; it may be likely that such mechanisms may be responsible for the differing antimicrobial susceptibilities. Notably, however, and in line with other antibiotics tested in the study, we found that synergy was present when aztreonam was tested in combination. This synergy was observed across the entire spectrum of species groups, though as stated elsewhere in this study, it is difficult to accurately predict whether

the combination of two poorly active antibiotics would add up to a more positive clinical outcome than the use of a single or groups of far more effective agents.

7.3 Mechanisms of synergy

It is difficult to account for the sporadic nature of synergy or antagonism. Indeed, where previous work has been carried out to determine synergy, antagonism or additive effects, there is very little information relating to the mechanisms behind these interactions. Many authors point out the clinical benefits or otherwise of the interactions, but do not suggest the basis/origin of the interactions. It is clear that fuller understanding of this is needed before a true picture emerges that can be reliably used in a clinical setting. Some combinations are known to act in a positive way (such as penicillins and amino-glycosides) and the mechanism is largely understood. One can only postulate that, as with the previous example, one of the antimicrobials is able to prepare conditions that are conducive to the next antimicrobial agent. Combinations where macrolide antibiotics (not tested here) have been included, for example, have been demonstrated to reduce biofilm/alginate production in CF isolates (Saiman *et al*, 2002). This is an example where the antibiotic used in combination is not necessarily acting upon the target organism, but may be simply making the environment around the organism more conducive to the bactericidal or bacteriostatic effects of the antibiotic given/tested in combination.

In terms of both the usefulness of synergy testing and therapy, and the scientific basis for carrying out these tests, it is interesting to note previous findings when *P. aeruginosa* was tested against combinations. It was found by Saiman *et al* (2002) that CF strains responded better to combination therapy than their non-CF equivalents. Equally, non-mucoid strains did not respond as effectively as the mucoid strains. This suggests that synergy testing may have some measurable, and tacit, benefit in those cases, and that the mechanisms of synergy

may not be straightforward. The possibility that synergistic combinations may act upon other bacterial/host factors in addition to the published bactericidal/bacteriostatic effects cannot be ruled out and would be an interesting area for further study.

Further supporting the idea of a cascading "open door" interaction, are other studies using non-antibiotic substances that considerably increase the efficacy of antibiotics when given in combination. One such study used membrane-active peptides (MEPs) that are capable of disrupting, with catastrophic effect the bacterial membrane, in combination with a number of commonly used antibiotics to test their effect against *S. maltophilia*. Given that the impermeability of *S. maltophilia* (and, indeed, other CF organisms) acts as a major innate resistance mechanism, it is thought that these MEPs are able to penetrate the cell and disrupt to an extent the peptidoglycan by the induction of bacterial hydrolases. This renders the bacterial membrane more porous and promotes synergy with some antibiotics (Giacometti *et al*, 2000). Once again, this demonstrates where a substance may be used to simply "open the door" prior to the bactericidal work of the active antimicrobial where this would not have been possible with only one antimicrobial alone. It must be stated, however, that the combination given above, was active only for a limited group of antibiotics – namely clarithromycin, β-lactams and polymixin B.

Despite the lack of an inhibitor, ceftazidime in this study was relatively successful when paired with tigecycline. Given the completely different mechanisms of action of these antibiotics, it may be possible that a cascading effect may have occurred. Poor permeability is a noted resistance mechanism in most CF related organisms – and particularly where the *Bcc* species are concerned. The presence of ceftazidime, as an antibiotic that damages the cell membrane, may be a factor in the further success of tigecycline. Given that tigecycline does not appear to be as efficacious when compared with minocycline – and this may be due to a difference in cellular penetration, it may be possible that the disruption of the cell

membrane may be sufficient to allow the further penetration of tigecycline – resulting in a synergistic effect. Furthermore, since synergy appears to be a phenomenon that is related to resistance (most occurrences of synergy were noted in otherwise resistant antibiotics), it is possible that the ceftazidime does not actually kill the cell, but simply acts as a conduit through which tigecycline can penetrate and disrupt the cellular protein production. This, of course, is speculation, and it is perhaps equally possible that the activity of tigecycline is sufficient upon the bacterial cell to disrupt the chromosomal β-lactamases – thus allowing ceftazidime to act more fully upon the cellular membrane structure.

It is clear from previous work that the mechanisms of synergy are poorly understood. Many clinicians, in fact, may see them as almost irrelevant so long as the outcome correlates successfully with the results of testing. A fuller understanding of these mechanisms, however, would allow this correlation to be understood further and it seems that further work is warranted in this subject.

The outcome of this study is difficult to judge objectively, as the measurement of outcomes may take a number of different forms. Highest on the list, of course, remains the clinical improvement of patients' health and wellbeing – especially those that are unfortunate enough to harbour resistant strains (McDowell *et al*, 2004). This study does not have a clinical remit and can only, therefore, act as a *predictive* indicator of success or failure. Only further trials *in-vivo* can accurately ascertain the relevance of the work carried out here.

Chapter 8

8.0 Relevance and application to practice

8.1 Clinical relevance of synergy testing and the application to practice of this study.

Previous work on synergy and combination antibiotic therapy has not always targeted the same organisms that were used in this study. The combinations used in this study are to a greater extent, unique at the time of writing. Interestingly, however, where data has been published we note that most studies have found similar levels of resistance *in-vitro* when carrying out MIC testing with the organisms used here. Likewise, we have also encountered similar levels of synergy, antagonism and indifference that, whilst sporadic and of a low level nature, is encouraging for further investigation (Saiman, 2002).

It is clear from the data that synergy does occur – albeit in a seemingly random and sporadic pattern. The data presented here, however, show that in most cases, synergy has occurred where the organism would have been otherwise resistant to the test antibiotic when tested individually. This poses an interesting question of whether this information is clinically useful/relevant. Where an organism was sensitive, then synergy may be somewhat less useful, since the antibiotic being tested would likely have yielded a positive clinical outcome anyway – with some exceptions noted earlier. It may be useful, in the reduction of resistance, and it could be argued that any improvement in MIC as a result of synergy could be beneficial. Where the organism was not susceptible *in-vitro*, however, it may be that a synergistic interaction would be sufficient to make the difference between a positive or negative clinical outcome – either by being sufficiently effecaceous in combination to kill the organism, or by rendering it vulnerable to host factors by damaging it. There are, of course, other issues that confound the results. CF isolates – especially Bcc organisms,

unlike *Pseudomonads* and other colonising organisms possess multiple resistance mechanisms that may not be overcome by simple combinations (hence there are now trials being carried out using triple combinations). One example of this would be where an aminoglycoside is used in combination with a β-lactam antibiotic. Under normal circumstances this combination is noted to produce a synergistic effect. With the Bcc organisms however, the presence of both chromosomally encoded β-lactamases, altered penicillin binding proteins, intrinsic resistance to aminoglycosides and other mechanisms such as antibiotic efflux pumps (effective against trimethoprim and ciprofloxacin), makes combination therapy an unpredictable and subjective method of treatment. Randomised control trials have found that triple combinations are generally beneficial in CF patients, though antagonism is common. This demonstrates a case for good quality synergy testing before treatment is commenced (Aaron *et al*, 2000). In Aaron *et al* (2000) the best bactericidal activity against *B.cepacia* was achieved using inhaled tobramycin, meropenem and a third antibiotic (ceftazidime, chloramphenicol, trimethoprim/sulfamethoxasole, aztreonam or amikacin). This suggests that where patients are stable, multiple combination testing can give an indication of likely therapeutic success in the event of an excacerbation. The benefit being that time is saved, and the patient will benefit from rapid targeted therapy (Aaron *et al*, 2000).

Where organisms are found to be resistant *in-vitro*, there is often still a positive response *in-vivo* when the patient receives antimicrobial chemotherapy. Likewise, antibiotics such as tobramycin and colistin have been demonstrated to be more useful in high doses (the most effective being colistin) – and in nebulised form, it is possible to reach these concentrations at the site of action. Without further clinical trials, it is difficult to predict the activity of tigecycline *in-vivo*, either alone or in combination when at higher concentrations for a number of reasons. Tigecycline is not currently available in a nebulised form and may only

be administered as an IV antibiotic meaning that the sites of administration will differ. The variable routes of administration along with variability in the organisms found in CF patients mean that the results of testing are difficult to predict with any accuracy. It is likely that this variation would carry over to the clinical treatment/response and would have an impact upon outcomes. It may not be possible to reach the concentrations required at the target site without administering higher doses, possibly over different timescales – indicating a higher cost in clinical contact. Issues of toxicity fall outside the remit of this study and would have to be investigated further. It should be noted, however, that this study has demonstrated that many isolates show intermediate sensitivity (or resistance) and that this can be viewed as a subjective end point of MIC testing. Where this occurs, there is a possibility of the antibiotic being successful in treatment of colonised patients – particularly those that are colonised with Bcc species. Likewise, given that we have demonstrated some synergy with other commonly used antimicrobials, it may be possible that some increased effectiveness will be seen when these combinations are attempted *in-vivo*. The real issue with these assumptions, however, is that the "hit rate" is relatively low. We were not able to demonstrate synergy across the whole spectrum of organisms, antimicrobials or, in fact, sensitivity in many cases. If the clinician were to rely upon the synergistic effects of antimicrobial combinations as tested here for empirical treatment, then failure is a significant possibility. As synergy was found to be in the order of 20% or less across the board, then we can assume that only a small percentage of these patient isolates will actually respond *in-vivo*. This makes the synergy testing versus clinical outcome link potentially tenuous. Conversely, when previous work is taken into account, we find that many studies that look at the potential clinical correlation between synergy testing and outcome do not take note of the small number of patients who have failed previous empirical treatments – and it is possible that this small subset of patients may benefit from such therapy. The end

point of treatment in CF as mentioned previously is not complete eradication. This would be physically impossible with current medicine. Reductions in colonisation with a commensurate improvement in symptoms is one of the primary goals. One aspect that many researchers do not appear to take into account is this slightly more limited – though just as important role. It doesn't stop there, however. Antibiotic treatment can have many benefits – they challenge or stress the bacterial cell sufficiently to alter its behaviour and this can be observed where multiple phenotypes are expressed. The reduction of biofilms, for example, may be a useful product of antimicrobial chemotherapy with the resulting drop in pathogenicity/virulence of the target organisms. It is in these specific areas that antibiotics may be useful – regardless of sensitivity results.

Given the nebulous occurrence of synergy and antagonism in all of the antibiotics tested in this study, combined with the doubt about the clinical uses of this data, one must pose the question about routine laboratory testing for synergy and its validity/usefulness. Many researchers appear to have taken a purely research/laboratory view of antimicrobial testing. Models such as the biofilm method used by Keays *et al* (2009) or, indeed the synergy model used in this study (Pankey & Ashcraft, 2005) may not lend themselves to the routine laboratory where thousands of CF related specimens are received every year. To carry out a simple disc test using any one of the currently available methods (modified Stoke's, BSAC), takes a very short period of time, and is relatively inexpensive. Most laboratories use this type of testing as a routine method, and though it does not give precise MIC data, it is predictive of outcomes. MIC testing is expensive and more time consuming than standard disc diffusion methods. Additionally, this expense is multiplied where numerous antibiotics are to be tested – either individually or in combination. To test in combination, as seen in the method section, one is required to test all of the antibiotics individually first – at considerable expense (over 24 h), followed by combination testing. The total time from a

fresh, pure culture, to a completed result is around 24 h for MIC testing and 48 h for a synergy test. It must also be taken into account that these organisms often do not oblige by growing in pure cultures, so additional time must be taken to purify organisms, thereby ensuring valid results. The technical time required of scientists in the laboratory is also greatly enhanced where synergy testing is used – taking up to 1.5 h to set up a small number of synergy tests. This is due to the long incubation required for diffusion. It is, therefore, unlikely that this type of testing will ever be considered for routine use within the laboratory. Newer suggested methods of testing may be more representative of the *in-vivo* response, but would also be more difficult to introduce into the routine laboratory. In the LTHT, specimens received number in the thousands, and it would be very difficult to conduct biofilm testing on each of these. Additionally, where multiple morphotypes are observed, this would considerably increase the cost and workload per patient. It remains to be seen how such methods could be introduced in a practical and inexpensive way (Keays *et al*, 2009).

Synergy testing is expensive and time consuming. A number of laboratories have been set up around the world (UK, USA, Canada, Australia and Europe) and the cost of testing single isolates can be as high as $300 (Aaron, 2007).

Many authors have questioned the clinical relevance of synergy and MIC testing and clearly have some distrust/doubts about how results of this should be employed in clinical practice. This study, unfortunately, has probably added little to this ongoing debate. It is possible to view the situation from two distinct positions. The first is that MIC/synergy testing is a complete waste of time and resources. The tests may be seen as being carried out in near "perfect" conditions, on a solid media with pure organisms and at concentrations that do not necessarily represent those found *in-vivo*. There are no host factors, the environment is carefully controlled and any effects are timed carefully. Compared to the human

environment where host factors abound, temperature and pH may vary considerably, and other drug-drug interactions make treatment unpredictable, the situation may be seen as quite different. Adding to this are the complications of multiple morphotypes, biofilm production, differential growth phases and the protective element that they provide. From this view, and it is quite understandable, clinicians may decide that empirical treatment can only be guided by clinical response and prior experience. Indeed, there are a great many previous studies that have found no correlation between testing, and clinical outcome (Smith *et al*, 2003; Aaron *et al*, 2006; Aaron, 2007; Etherington, 2007; Fowraker *et al*, 2009; Fowraker *et al*, 2010).

The other viewpoint contends that MIC and synergy testing may actually be a useful tool where used properly – and it is this *appropriate* use that is advocated here. As with any scientific methodology that informs clinical treatment, the response of the patient is the end point and the *raison d'etre* behind all of the work – including this study. If the data are used appropriately, with the previously mentioned limitations taken into account, then we believe that MIC and synergy testing may be a useful, and perhaps powerful tool to guide empirical treatment of this unfortunate group of patients. Where an organism (including multiple morphotypes) has been identified and tested, then it should be remembered that this is the same causative/colonising organism possessed by the patient. *Some* of its response, therefore, will be representative of the likely response *in-vivo*. Where options are limited by resistant organisms, multiple morphotypes and phenotypical expression, there are only two avenues of action. Empirical therapy guided by experience, and evidence based targeted therapy. Despite obvious limitations of MIC/synergy testing, it stands to reason that this must still offer a more accurate view of likely outcome than simple guesswork – though experienced clinicians may be able to predict the likely response for fast treatment.

The position taken by this study – based upon the evidence gathered here, is that on the whole, synergy testing is not useful. This is because it is time consuming, expensive, and in most cases – as seen here, will not yield any useful information. The vast majority of antibiotic combinations that are tested result in indifference. There are situations, however, where a patient suffering from an exacerbation or significant morbidity may benefit from the small changes in efficacy that synergistic combinations may provide. As stated earlier in this study, there have been noted benefits of combinations in particularly sick patients – but these could not be correlated with testing. This does not mean that there is no correlation. In those vulnerable, perhaps, last chance patients then synergy may be warranted. Given the heterogeneous nature of both the organisms, and the occurance of synergy/antagonism, it is essential that combinations should be trialled in-vitro first, to prevent clinical failure.

Where CF patients have undergone lung transplantation, their risk of death within 1 year of this procedure has been noted to increase by up to six times. Any reduction in colonisation has to have significant benefits for these risk groups and cannot be underestimated (Alexander *et al*, 2008). Further benefits of the successful reduction in numbers of patients colonised with *B.cenocepacia* and, indeed Bcc generally, include the not inconsiderable improvement in psycho-social status of patients that attend the CF centre. It is clear that the isolation of patients that harbour these strains can have a dramatic effect upon their social lives and the way in which they are accepted by other CF patients that may attend the same unit or other CF related events. The reduction in the prevalence of Bcc strains in particular, will reduce this level of segregation for patients and improve their psycho-social wellbeing. More difficult to measure, are the spin-off benefits of this – such as the improvement in health that is often observed in "happy" patients (Speert *et al*, 2002; McDowell *et al*, 2004). A further benefit of such improvements can be seen in the microbiological monitoring that is required. Isolates such as the Bcc species require complex identification at specialist

reference centres. A general reduction in the isolation of these organisms from patients attending the CF unit may have a commensurate reduction in spending as a result of this drop in identification referrals in some units, though the LTHT currently does not pay for these. Normal microbiological monitoring, however, is unlikely to change as a result of this, as it is important to maintain a clear picture of each patient's colonisation status (McDowell *et al*, 2004). Additionally, it should be noted that the isolation procedures of any CF unit will not mitigate against the sporadic spread of Bcc from environmental sources – meaning that expenditure on screening remains prudent (McDowell *et al*, 2004).

We have demonstrated in this study that tigecycline has disappointing activity against the Bcc organisms. Equally, we have demonstrated that other antimicrobials are still effective – and that there are still many options with each of the species tested. We have demonstrated that synergy does exist, it does occur with most of the groups tested, and that multiple combination therapy can have beneficial effects – though it should be used carefully, and after thorough testing and review of individual cases. It may be that synergistic combinations of antibiotics will be the way forward as options are limited by further resistant phenotypes. Only further, thorough, study will provide the full answer as to the correlation between testing and outcome.

8.2 Limitations of this study and suggestions for further work

As with many studies carried out in the pursuit of novel treatment strategies for patients with difficult conditions, there are a number of limitations and confounding factors that can affect the outcome of such work. This is particularly true for CF patients and their associated isolates and antibiotics. It is appreciated that the list of limitations given here is somewhat long and may cast some doubt upon the likely success, or impact of this study, though these confounders are quite normal and common in a CF study of this size. Many of the issues

highlighted fall outside the remit of a simple *in-vitro* investigation such as this one, and require huge budgets, ethical approval, willing patients and time to complete – often making them impractical on a modest budget. Any such approach would have to include multiple centres – both in the laboratory and clinical environment.

One issue that remains unresolved by this study is that of *clinical* efficacy of the antibiotics tested – both alone and in combination. Whilst we can postulate based upon *in-vitro* data, it is difficult to fully understand the *in-vivo* relationships between bacterium, host and antimicrobial. Tigecycline is clearly less effective – particularly where Bcc species are to be the target organisms, though it could be that *in-vivo*, the effect of host factors, biofilm growth and antibiotic may change this relationship sufficiently that this balance is redressed. A study by Keays *et al* (2009) took a slightly different approach to susceptibility testing – and this may further develop the methods used in this study. They chose to use a biofilm model, citing the build up of biofilm in CF patients as a potential confounding factor. Their model was used to test susceptibilities of the bacteria that are tied up in a biofilm and, therefore, likely to be exhibiting altered phenotypical expression – resulting in differing antibiotic susceptibilities. They had found that antibiotics had previously been prescribed based upon culture results from planktonically grown bacteria. Unfortunately, isolates grown as planktonic cultures were found to be far more susceptible (60%) to previously prescribed antibiotics than the same organisms grown in a biofilm model (22%). How this correlates to clinical outcomes, however, remains to be seen. In the study, they used multiple parameters to calculate clinical outcomes, and though this study largely stands by itself in its methods, it may inform future work and, indeed, be more representative of the situation found in CF patients *in-vivo*. Another aspect of growth in biofilms that is often not taken into account, is the growth phase of the organism at the time of testing. How this information could be introduced into testing is difficult to imagine, but a study by Desai *et al*

(1998) demonstrated that this can make a significant difference to the results of susceptibility testing. Biofilm susceptibility testing appears to be one way that susceptibility may be ascertained, though this is, as yet, a new idea and has not been thoroughly correlated with clinical outcomes or more standardised methods (Caraher *et al*, 2007; Keays *et al*, 2009).

One important aspect of susceptibility testing in CF patients that may be overlooked, is that it does not often correlate to a specific end point (Foweraker *et al*, 2009). Where antibiotics may be used in a case of bactaeremia, for example, the end point is the eradication of the target organism. This can be measured with some accuracy. In CF patients, however, the aim is for a *reduction* in bacterial colonisation and the resulting improvement of pulmonary symptoms. Eradication is not only unlikely, it is virtually impossible to clear the lower respiratory tract of organisms. It is reasonable to assume, therefore, that such open ended susceptibility testing may not correlate accurately with outcomes. There are also many ways in which these improvements can be measured – from improvements in forced expiratory volumes, to the wellbeing of the patient. Further work to take into account these factors in combination with multiple antibiotic treatments correlated with MIC/synergy testing would possibly answer some of the questions relating to clinical efficacy and outcome. It seems likely, however, that where studies take into account multiple factors, they will likely arrive at different conclusions based upon the factors chosen (Foweraker *et al*, 2009). Clinical outcome is difficult to measure, and to include this in any study of this type would substantially increase the difficulty by the addition of a number of confounding factors that would have to be carefully controlled such as environmental factors, host factors, concurrent treatments etc.

Possible reasons that combination testing may not yield a definite clinical benefit when compared to single antibiotic testing are unclear. Other authors have investigated this

phenomenon, and numerous comments have been made all of which may be relevant to this study (Aaron et al, 2005). As with many other studies carried out, we were unable to account for "unknown" confounders. An example of this can be seen where apparent synergy occurs but where this is not translated into clinical benefit in a controlled trial. The possibility that some antimicrobials (Aaron *et al* (2005) use the example of azithromycin)) may have other effects that are of more benefit in improving clinical outcome (such as the reduction of inflammation) – whereas the enhanced bactericidal effect produced by synergistic interactions may make little difference against a large and variable population of resistant organisms.

Given the differences in expression of antibiotic susceptibility of organisms investigated in this study, it may have been prudent to log concurrent antibiotic treatment of patients from whom the isolates were obtained. Having demonstrated links between previous treatment and the expression of resistance (Drevinek *et al*, 2008), it would follow that a prospective study may shed further light onto the clinical relevance, impact and after effects of treatment with the antimicrobials available. Unfortunately, prospective studies to assess the correlation between antimicrobial sensitivities and clinical response are difficult to carry out due to the ethical dilemma of treating/not treating where susceptibilities suggest otherwise. We maintain, therefore, that only retrospective data can be used and may be flawed by other confounding factors that are not recorded concurrently. For future work, a retrospective study taking into account all of the above may yield more accurate and clinically relevant data. This would be time consuming and even in a busy CF unit, reliable data utilising sufficient numbers of isolates and patients would take some considerable time to collate.

Detection of higher levels of synergy may not be possible due to limitations in the E-test. Because there is a lower limit to the E-test antibiotic strip, low MIC organisms may grow close to, or even exceed these limits – giving no discernable ellipse difference. Where

synergy occurs at or around this point, we would simply not detect it. Likewise, at high MIC values we would have similar problems – and this may skew data relating to high concentration antibiotics where they are administered by nebulisation. In addition to this, we did not grow or detect SCVs of any organism type – possibly due to the reliance on a solid culture medium. In mitigation of this, the final MIC/synergy test results would be a composite of any isolate plus its SCV and so we consider the MIC/synergy results to be accurate.

One potential problem with antimicrobial therapy for CF patients is the relative promiscuity of the organisms with which they are colonised. Previous studies have demonstrated that where an exacerbation occurs, treatment is likely to be given. In most clinical situations, this will be informed by previous microbiological results. Where there has been any delay in real time between testing and the exacerbation (a 23% difference in susceptibility was found in a study conducted by Aaron *et al* (2005) where treatment was given 3 months after the last susceptibility test), there is the strong likelihood that antimicrobial susceptibilities may have evolved and that previously efficacious antibiotics may be rendered useless.

There are currently no published MIC values against the Bcc species and other organisms in this study – particularly in relation to the tetracycline class of antimicrobials. The result is that we have extrapolated data from the nearest and most similar organisms – in most cases *P.aeruginosa*. Until guidelines are published, we can only speculate about any error that arises as a result of these assumptions, and at present, the data can only be taken at face value. Of note, is that other authors who publish similar data have access to the same BSAC/CLSI data that was used here, and so we can say with some confidence that our data is standardised with any concurrent works.

In calculating synergy, we described our data as “synergistic”, “antagonistic” or “indifferent”. It was noted that previous studies have also used the term “additive” where a small proportion of those results falling between Synergistic and antagonistic could be described to represent the sum of the two susceptibilities (Saiman *et al*, 2001). It was decided for the benefit of this study to ignore this term, since it is not only difficult to accurately ascribe an additive vs synergistic effect (where summative results occur, we cannot say exactly how this has occurred), but may be clinically irrelevant. If two antibiotics are found to inhibit growth, and they show “indifference” when paired together, then clinically, it may be assumed that no antagonism will occur. The likely result, therefore would be either additive or, indeed, indifferent where the combination is used. In the interest of brevity, therefore, and to avoid producing misleading results in what is already a difficult clinically quantifiable situation, the term “additive” was omitted from this study. For completeness, however, it should be noted that an additive effect would generally be considered to include those organisms with a summation FIC of 0.5 - < 1.0 (Saiman *et al*, 2001).

It has been demonstrated that environmental colonisers such as *Pseudomonas* and *Burkholderia* are often present in multiple morphotypes – each with a different bacterial sensitivity. It can be very difficult, therefore, to obtain consistent and repeatable MIC data for these organisms as each subsequent testing may select another colonial variant that, to the microbiologist, appears to be the same as the rest (Doring *et al*, 2000).

Given that minocycine is the legacy analogue of tigecycline and was shown, overall, to be more effective *in-vitro* than tigecycline, it may have proved beneficial to attempt synergy testing with this antimicrobial.

Chapter 9

9.0 Final Reflections

I write this, a short piece of reflection, as the project nears its conclusion after several years with the benefit of hindsight and humility. It is easy, within the scientific profession of microbiology – and I would imagine other professions, to become somewhat closeted – not just in our own specialities, but to a degree within our own world and self importance. Viewing the NHS and the world through the eyes of a microbiologist is comforting in its familiarity. This project has challenged this familiarity in a number of ways – few of which I expected. Of course, all soldiers like to play the part of generals; nurses second guess doctors, and scientists claim to know the secrets of life, the universe and everything. How humbling, therefore, to work outside our own normal remit – inductively learning and updating the process of research.

9.1 Personal development and learning

In a moment, perhaps a year or so ago, I understood how the astronauts of the Apollo mission must have felt as they viewed the blue orb of Earth from afar – tiny in our almost infinite universe. It was this moment in which the true extent and reach of projects such as this became apparent. The realisation that the science, whilst important, plays but a small part in the whole clinical process was something of a revelation. I've always been aware of the wider implications of the biomedical profession – one must always bear in mind that a life and its welfare is the end point of anything that we do on a day to day basis. Mistakes can sometimes be costly. Working against this, however, is the day to day routine. Tests arrive in the laboratory. We perform them, and then release the results – where some distant and anonymous clinician interprets them and treats the patient. We exist in this comfortable

world from day to day – and only occasionally step out into the wider world of clinical medicine. It would be true to say, without being disparaging of the diagnostic scientist that there is very little reflection on or in action during these processes (Schon, 1987).

This project has been my journey into that wider world. Ironically, it has been a journey into a small part of the wider world – being specialised to such a degree in CF medicine.

I think it is fair to say that my own learning style throughout this project has been somewhat inductive. I was given the idea and brief by Dr Miles Denton – a consultant clinician and specialist in CF medicine. We had some idea of the likely outcome – previous work had been carried out on these organisms, though it was limited. My own position was largely technically based – culturing bacteria and interpreting sensitivity patterns. Looking into the wider field of clinical medicine took some adjustment – not only in my own knowledge and scope of practice, but also in interpretation of the science. Much of the process during this project – particularly during the first 3rd was somewhat inductive and reflects the "self directed learning" style suggested by Gerald Grow (1996). This was not what I had at first anticipated, but with the benefit of some hindsight, I believe that the project is richer in its content, and I feel that I have more ownership than if the whole project had been dictated. Dr Miles Denton was very supportive of this method of study and has been a constant source of contact where questions arose. It is this support that ensures success as suggested by Daloz (1986) in a model where mentorship must match the challenge. Where the challenge is high and mentorship is low – so failure is likely.

The work that I carried out for this study was practically based – well within my own comfort zone. The difference on this occasion was the reflection on action that was required. Not only did I have to look at the work that I was carrying out – was it done accurately/correctly and was it appropriate, but also how did it fit with the literature and

current thinking in CF medicine. This kind of reflection suggested by Schon (1987) is one that research scientists likely perform without being aware. For the diagnostic scientist, however, where results are produced, released and the next test arrives, it is more unusual. It took me some considerable time to change from an almost "concrete" style of reflection upon action to a more "formal style" (Dembo, 1994) where every action carried out had to be analysed and put into context with the wider field of clinical medicine. With a more "formal" style, it was possible to find the meaning of the work that was being carried out.

In Fig 9.1, my own learning style during this project is represented. It appears somewhat complex, but for the user – me, it is remarkably simple: the usual reflection takes place – looking at an event, that has just occurred about which I am not content (or, in fact, it may be a positive event which I choose to look back on and learn what I did right). My feelings are explored – was it painful, embarrassing, should I feel contented/proud of the achievement, followed by a rational look at my own position - did I perform the task accurately, and *at what level*? Am I competent or proficient – do I work according to the standard operating procedure, or am I able to use my own clinical judgement? Here, there is the option of mentoring – someone to tell you at what stage you appear to be. It is or at least should be, however, a two way process (Johns, 2000). At some stage, you have to look forward – what am I going to do next to solve the problem or build upon a success? Again, in the right situation, a mentor can help, if appropriate. This reflection continues with constant reference to a fixed point – where am I now, at what stage in my research and competence (this can be illustrated by the ladder of competence suggested by Dreyfus & Dreyfus (1986). This project has been pieced together – rather in the style favoured by Teekman (2000) where any piece of work, event or thought process can be broken into discrete portions. There were a number of distinct portions to this project – each with their own challenges and response – and each in-turn requiring a different, reflective approach.

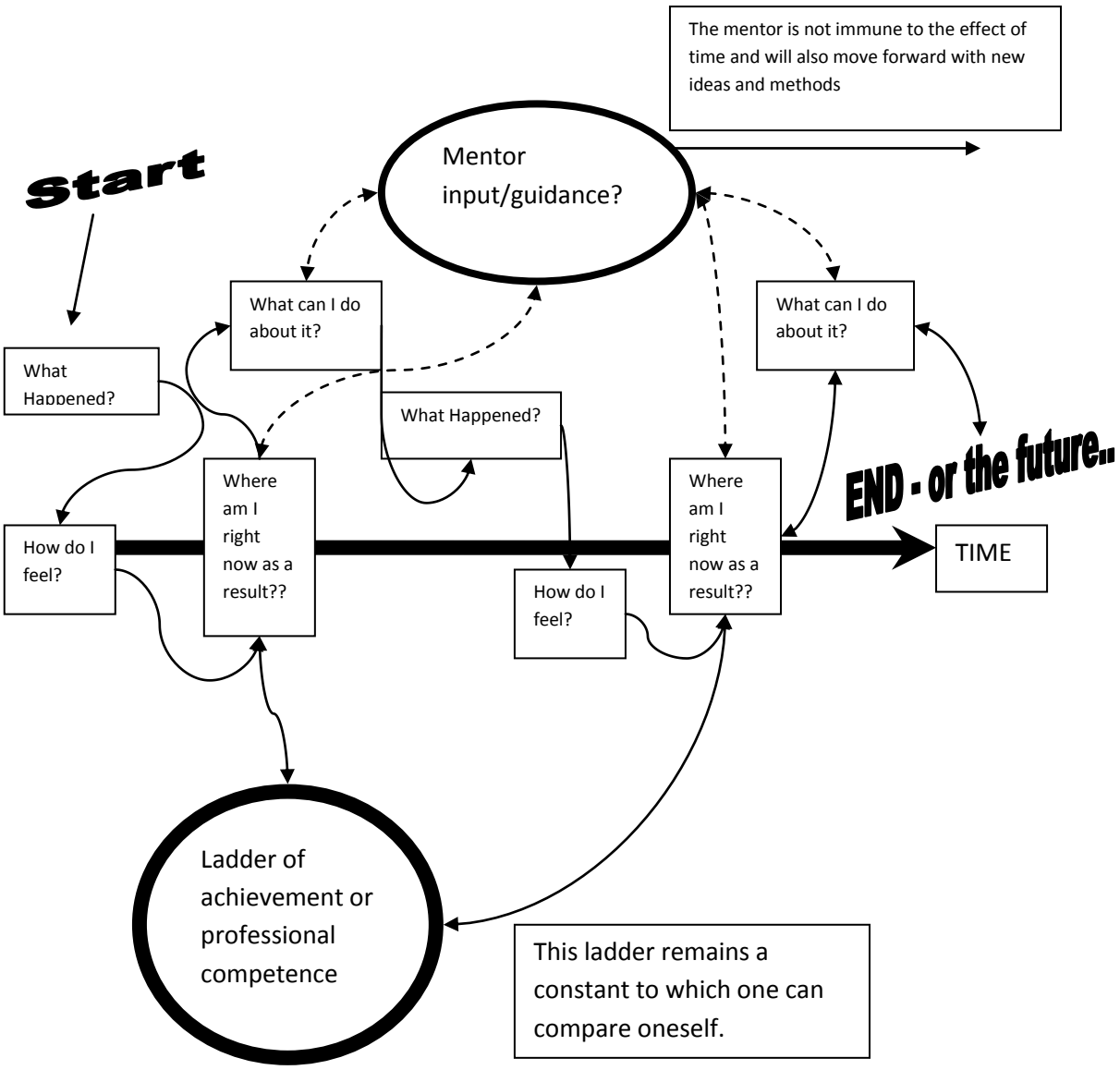

Fig 9.1: The reflective process of this project.

These ultimately have combined to produce the final thesis presented here. I think that this was a necessary process. The large size of this document along with the complexity of balancing work and play, have made it very difficult to see the project as a whole – particularly during the writing phase. By breaking down the project, it has been possible to give each section the attention that it was due – in the hope that the whole thing would fit together well. It was this final stage that was the most difficult and having asked a number

of completely neutral people to read the document, could I be assured that it works as I hoped.

My time at during this project fulfilled several criteria that are set down by Carper (1978) when several patterns of "knowing" were identified.

Empirical knowledge - was the first of these fundamental criteria to be addressed. I was able to increase my theoretical and technical knowledge about CF.

Personal Knowledge - learning about oneself and how to interact with other professionals or patients. Each of the parts of this project involved interactions with different groups. And each part as it became complete, gave me further confidence and knowledge to move onto the next.

Aesthetic knowledge - a subjective and tacit knowledge gained through experience does not come quickly and I think it would be safe to suggest that I still have much to learn. In a profession such as the biomedical sciences, tacit knowledge is less appropriate although is still used by the proficient professional (Dreyfus & Dreyfus, 1986). Scientific fact is the main driving force behind decisions that are made – and this fits nicely with the latest evidence based practice campaign of the NHS (Todd *et al*, 2004). Because we do not deal directly with patients and make judgements based upon feelings, it has taken time to gain this kind of knowledge.

I think that the process of reflection during this project has been complex and multi-faceted. Learning wasn't the only issue, and neither was my own technical expertise as a scientist. The fear of failure and the desire to be a better professional was, perhaps, the overriding motivation during this project – and at times the only thing that stopped me from failure.

It is, perhaps, poignant that one should write a reflective piece about this large academic project. I think that with a busy life encompassing both a new baby, and demanding job along with steadily advancing years, this is likely to be my last substantial academic piece of work. It has in some ways been a cathartic experience – highlighting new areas of deficiency and putting to rest others. I sincerely hope that whilst the clinical benefit of this project was, perhaps minimal; it may somewhere fit into the larger jigsaw and ultimately benefit those unfortunate sufferers of CF.

9.2 Publication and dissemination

One unfortunate part of this project – and no doubt a similar issue to all such research ventures was its end point. Such ventures hinge upon results, and as such this can often determine whether it is viewed in a positive or negative light.

Tigecycline is, of course, not just an antibiotic. It is a commercial product, and as such it exists in the commercial world to be sold and to make profit. Having gained an unrestricted research grant from Wyeth Pharmaceuticals for this project, it was natural that they would wish to publicise the work that was carried out. I had some initial misgivings about this – wondering if the company would wish to put its own version of events forward, but these were soon alleviated when we had no such restrictions either suggested or imposed. To this end, I accompanied a group of sales representatives to the European Conference of Microbiological and Infectious Diseases (ECCMID) where we presented a poster that was prepared by Dr Miles Denton (project supervisor) along with an abstract (Appendix 2). In addition to this, a presentation was made at a CF conference in Brest, France by Dr Denton.

Whilst this met with some success, as we were able to point out the relative benefits and limitations of tigecycline against specific organisms, we were unable to proceed further with publication. The reasons given for this were numerous – some due to technicalities in the

way in which we had prepared the data though ultimately, it was felt by both the *Journal of Antimicrobial Chemotherapy* and *Journal of Cystic Fibrosis* that our data did not have a sufficiently positive clinical impact to warrant further publication. Whilst I do not agree on this point – contending that we have demonstrated not only where testing is not appropriate, but also where it is, perhaps, entirely appropriate - this was a major blow.

It was noted by Boyd & Fales (1983) that negative experiences are one of the strongest driving forces behind reflection and change – and this was true to an extent in the way that this project was carried out. From this point, we were able to identify not only where the strengths of the project were, but also its weaknesses. The main problem was that correlation of our results into clinical success is very difficult to achieve with CF patients. I believe that this has become an important factor in our research and that any future attempt at publication would have to cover this subject in some depth. In the meantime, this research thesis highlights many of these issues and is likely to be of use in the LTHT and hopefully, as other researchers pick up the loose ends, in the wider field of CF medicine. My feeling, based upon experience within the LTHT and other microbiology departments, is that this work will not be wasted by the limited exposure it has received. Ultimately, it is likely that other projects, perhaps on a smaller scale, will incorporate these findings and expand upon them.

Unfortunately, it has been said in the past that "science does not exist until it is published" (Garcia, 2004). We must, though, at this stage take comfort in the positive effect that was gained through the poster presentation and literature produced by Wyeth pharmaceuticals. Publication and dissemination has occurred for this project – just not as widely as we would have hoped. Though the clinicians in the LTHT CF unit, the findings of this project will find an audience and may, indirectly or otherwise, have some benefit howsoever it occurs. My feeling, is that publication will occur eventually, but that there may be some challenge in

persuading non-CF clinicians and researchers of the value of this work since they mainly work in finite end points – eradication of infection and curing patients. It is an unfortunate aspect of CF medicine that we can say both as researchers, scientists or clinicians that this is not currently possible with available medicine. For my part, I hope that this work benefits patients in the LTHT, and through the steady dissemination however it occurs, a wider population of CF sufferers.

10.0 References

Aaron, S. D. (2007). Antibiotic synergy testing should not be routine for patients with cystic fibrosis who are infected with multi-resistant bacterial organisms. *Paediatric Respiratory* R*eviews,* 8, 256-261.

Aaron, S. D., Ferris, W., Henry, D. A., Speert, D. P., & Macdonald, N. E. (2000). Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with *Burkholderia cepacia*. *American Journal of Respiratory Critical Care Medicine*,161, 1206-12.

Aaron, S. D., Vandemheen, K. L., Ferris, W., Fergusson, D., Tullis, E., Haase, D., Berthiaume, Y., Brown, N., Wilcox, P., Yozghatlian, V., Bye, P., Bell, S., Chan, F., Rose, B., Jeanneret, A., Stephenson, A., Noseworthy, M., Freitag, A., Paterson, N., Doucette, S., Harbour, C., Ruel, M., & MacDonld, N. (2005). Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria: a randomised, double-blind, controlled clinical trial. *The Lancet,* 366, 463-471.

Alonso, A., & Martinez, J. L. (1997). Multiple antibiotic resistance in *Stenotrophomonas maltophilia*. *Antimicrobial Agents and Chemotherapy*, 41, 1140-1142.

Alexander, B. C., Petzold, E. W., Reller, L. B., Palmer, S. M., Davis, R. D., Woods, C. W., & LiPuma, J. J. (2008). Survival after lung transplantation of cystic fibrosis patients infected with *Burkholderia cepacia* complex. *American Journal of Transplantation*, 179, 1025-1030.

Alonso, A., & Martinez, J. L. (2000). Cloning and characterisation of SmeDEF, a novel multidrug efflux pump from *Stenotrophomonas maltophilia*. *Antimicrobial Agents and Chemotherapy,* 44(11), 3079-3086.

Anderson, D. H. (1938). Cystic fibrosis of the pancreas and its relation to celiac disease. *American Journal of Diseases of Children,* 56, 344-399.

Anderson, D. J., Kuhns, J. S., Vasil, M. L., Grding, D. N., & Janoff, E. N. (1991). DNA fingerprinting by pulsed field gel electrophoresis and ribotyping to distinguish *Pseudomonas cepacia* isolates from a nosocomial outbreak. *Journal of Clinical Microbiology,* 29, 648-649.

Anderson, S. W., Stapp, J. R., Burns, J. L., & Qin, X. (2007). Characterization of small colony variant *Stenotrophomonas maltophilia* isolated from the sputum specimens of five patients with cystic fibrosis. *Journal of Clinical Microbiology,* 45(2), 529-535.

Anonymous (2005) Instructions to authors. *Antimicrobial Agents and Chemotherapy,* 49, 1-20.

Baldwin, A., Mahenthiralingam, E., Drevinek, P., Pope, C., Waine, D. J., Henry, D. A., Speert, D. P., Carter, P., Vandamme, P., LiPuma, J. J., & Dowson, C. G. (2008). Elucidating global epidemiology of *Burkholderia multivorans* in cases of cystic fibrosis by multilocus sequence typing. *Journal of Clinical Microbiology,* 46(1), 290-295.

Balke, B., Hoagardgt, M., Schmoldt, S., Hoy, L., Weissbrodt, H., & Haussler, S. (2006). Evaluation of the E-test for the assessment of synergy of antibiotic combinations against multi-resistant *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *European Journal of Clinical Microbiology and Infectious Diseases,* 25(1), 25-30.

Belkin, R. A., Henig, N. R., Singer, L. G., Chaparro, C., Rubenstein, R. C., Xie, S. X., Yee, J. Y., Kotloff, R. M., Lipson, D. A., & Bunin, G. R. (2006). Risk factors for death of patients with cystic fibrosis awaiting lung transplantation. *American Journal of Respiratory and Critical Care Medicine*, 173, 659-666.

Berg, G., Roskott, N., & Smalla, K. (1999). Genotypic and phenotypic relationships between clinical and environmental isolates of *Stenotrophomonas maltophilia*. *Journal of Clinical Microbiology,* 37(11), 3594-3600.

Bernhardt, S. A., Spiker, T., Coffery, T., & LiPuma, J. J. (2003). *Burkholderia cepacia* complex in cystic fibrosis: frequency of strain replacement during chronic infection. *Clinical Infection and Disease*, 37, 780-785.

Biddick, R., Spilker, T., Martin, A., & LiPuma, J. J. (2003). Evidence of transmission of *Burkholderia cepacia*, *Burkholderia multivorans* and *Burkholderia dolosa* among persons with cystic fibrosis. *FEMS Microbiology Letters*, 228, 57-62.

Blazevic, D. J. (1976). Current taxonomy and identification of non-fermentative Gram-negative bacilli. *Human Pathology,* 7, 265-275.

Boyd, E. M., & Fales, A. W. (1983). Reflective learning: Key to learning from experience. *Journal of Humanistic Psychology*, 23(2), 99-117.

Brown, P., Butler, S., Nelson, J., Doherty, C., Govan, J., & Greening, A. (1993). *Pseudomonas cepacia* in adult cystic fibrosis: accelerated decline in lung function and increased mortality. *Thorax*, 48, 425-426.

Burkholder, W. H. (1950). Sour skin, a bacterial rot of onion bulbs. *Phyto-pathology*, 40, 115-117.

Bonacorsi, S., Fitoussi, F., Lhopital, S., & Bingen, E. (1999). Comparative *in-vitro* activities of meropenem, imipenem, temocillin, piperacillin, and ceftazidime in combination with tobramycin, rifampin, or ciprofloxacin against *Burkholderia cepacia* isolates from patients with cystic fibrosis. *Antimicrobial Agents and Chemotherapy*, 43(2), 213-217.

Bonapace, C. R. R., White, R. L., Friedrich, L. V., & Bosso, J. A. (2000). Evaluation of antibiotic synergy against *Acinetobacter baumannii*: a comparison with E-test, time kill and checkerboard methods. *Diagnostic Microbiology and Infectious Disease*, 38, 43-50.

Boyd, E. & Fales, A. (1983). Reflective learning: Key to learning from experience. *Journal of Humanistic Psychology*, *23*, 99-117.

Caraher, E., Reynolds, G., Murphy, P., McClean, S., & Callaghan, M. (2007). Comparison of antibiotic susceptibility of *Burkholderia cepacia* complex organisms when grown planktonically or as biofilm *in vitro*. *European Journal of Clinical Microbiology and Infectious Diseases,* 26(3), 213-216.

Carper, B (1978). Fundimental patterns of knowing in nursing. Advances in nursing science, 1 (1), 13-23

Ciofu O, Jensen T, Pressler T, Krogh Johansen H, Koch C, Hùiby N. (1996) Meropenem in cystic fibrosis patients infected with resistant Pseudomonas aeruginosa or Burkholderia cepacia with hypersensitivity to Beta lactam antibiotics. *Clinical Microbiology Infections*, 2, 91-98

Craig, C. R., Stitzel R. E. (2007) *Modern Pharmacology*. Lippincott Williams & Wilkins.

Daloz, L.A. (1986). *Effective Teaching and Mentoring*. London: Jossey-Bass.

David, E., Geller, M. D., & Rubin, B. K. (2009). Respiratory care and cystic fibrosis. *Respiratory Care*, 54(6), 796-800.

De Baets F, Schelstraete P, Van Daele S, Vaneechoutte M. (2007). *Achromobacter xylosoxidans* in cystic fibrosis: prevalence and clinical relevance. *Journal of Cystic Fibrosis 2007*; 6: 75-78

De Ley, J., Kersters, K., Khan-Matsubara, J., & Shewan, J. M. (1970). Comparative D-gluconate metabolism and DNA base composition in *Achromobacter* and *Alcaligenes*. *Journal of Microbiology and Serology,* 36, 193-207.

Denton, M., & Kerr, K. G. (1998). Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. *Clinical Microbiology Reviews,* 11(1), 57-80.

Denton, M., Todd, N. J., Kerr, K. G., Hawkey, P. M., & Littlewood, J. M. (1998). Molecular epidemiology of *Stenotrophomonas maltophilia* isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. *Journal of Clinical Microbiology,* 36(7), 1953-8.

Dembo, M. H. (1994). *Applying educational phsychology* (5th Ed.). New York: Longman.

Desai, M., Buhler, T., Weller, P. H., & Brown, M. R. W. (1998). Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during exponential growth. *Journal of Antimicrobial Chemotherapy,* 42, 153-160.

Dodge, J. A., Lewis, P. A., Stanton, M., & Wilsher, J. (2007). Cystic fibrosis mortality and survival in the UK: 1947-2003. *European Respiratory Journal,* 29, 522-26.

Doring, G., Conway, S. P., Heijerman, H. G. M., Hodson, M. E., Hoiby, N., Smyth, A., & Touw, D. J. (2000). Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a European consensus. *European Respiratory Journal*, 16, 749-767.

Drevinek P, Holden MT, Ge Z, Jones AM, Ketchell I, Gill RT, Mahenthiralingam E. (2008). Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when Burkholderia cenocepacia grows in cystic fibrosis sputum. *BMC Infectious Diseases*. 19;8:121.

Dreyfus, H. L., & Dreyfus, S. E. (1986). *Mind over machine.* New York: Free Press.

Eliopoulos, G. M., & Eliopoulos, C. T. (1988). Antibiotic combinations: should they be tested? *Clinical Microbiology Reviews,* 1, 139-156.

Entenza, J. M., & Moreillon, P. (2009). Tigecycline in combination with other antimicrobials: a review of in-vitro animal and case report studies. *International Journal of Antimicrobial Agents,* 34(8), 8.e1-8.e9.

Etherington, C., Hall, M., Conway, S., Peckham, D., & Denton, M. (2007). Clinical impact of reducing routine susceptibility testing in chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. *Journal of Antimicrobial Chemotherapy,* 61, 425-427.

France, M. W., Dodd, M. E., Govan, J. R., Doherty, C. J., Webb, A. K., & Jones, A. M. (2008). The changing epidemiology of *Burkholderia* species infection at an adult cystic fibrosis centre. *Journal of Cystic Fibrosis,* 7(5), 368-372.

Frederiksen, B., Koch, C., & Hoiby, N. (1999). Changing epidemiology of *Pseudomonas aeruginosa* infection in Danish cystic fibrosis patients (1974-1995). *Paediatric Pulmonology,* 8, 59-66.

Foweraker, J. E., Laughton, C. R., Brown, D. F., & Bilton, D. (2009). Comparison of methods to test antibiotic combinations against heterogeneous populations of multiresistant *Pseudomonas aeruginosa* from patients with acute infective exacerbations in cystic fibrosis. *Antimicrobial Agents and Chemotherapy,* 53(11), 4809-4815.

Foweraker, J. E., Laughton, C. R., Brown, D. F. J., & Bilton, D. (2010). Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. *Journal of Antimicrobial Chemotherapy*, 55, 921-927.

Gabriel, P. S., Zhou, J. Z., Tabibi, S., Chen Y., Trauzzi M., Saiman, L. (2004) Antimicrobial susceptibility and synergy studies of Stenotrophomonas maltophilia isolates from patients with cystic fibrosis. *Antimicrobial Agents and Chemotherapy*. 48 (1), 168-171

Garcia, A. M. (2004). Sixth version of the "uniform requirements for manuscripts submitted to Biomedical Journals": lots of ethics, some new recommendations for manuscript preparation. *Journal of Epidemiology and Community Health*, 58, 731-733.

Geller, D. E. (2009). Aerosol antibiotics in Cystic Fibrosis. *Respiratory Care,* 54(5), 658-669.

Giacometti, A., Cirioni, O., Del Prete, M. S., Barchiesi, F., Fortuna, M., Drenaggi, D., & Scalise, G. (2000). *In-vitro* activities of membrane active peptides alone and in combination with clinically used antimicrobial agents against *Stenotrophomonas maltophilia*. *Antimicrobial Agents and Chemotherapy*, 44(6), 1716-1719.

Gold, R., Jin, E., Levinson, H., Isles, A., & Fleming, P. C. (1983). Ceftazidime alone and in combination in patients with cystic fibrosis: lack of efficacy in treatment of severe respiratory infections caused by *Pseudomonas cepacia*. *Journal of Antimicrobial Chemotherapy,* 12(supplement A), 331-336.

Goldman, M. J., Anderson, G. M., Stolzenberg, E. D., Kari, U. P., Zasloff, M., & Wilson, J. M. (1997). Human beta-defensin 1 is a salt sensitive antibiotic in lung that is inactivated in cystic fibrosis, *Cell*, 88, 553-560.

Goldman, P. L., & Petersdorf, R. G. (1979). Significance of methicillin tolerance in experimental *Staphylococcal* endocarditis. *Antimicrobial Agents and Chemotherapy,* 15, 802-806.

Govan, J. R., Brown, P. H., Maddison, J., Doherty, C. J., Nelson, J. W., Dodd, M., Greening, A. P., & Webb, A. K. (1993). Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. *The Lancet,* 342, 15-19.

Govan, J. R., & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological Reviews,* 60, (3), 539-574.

Greer, N. D. (2006). Tigecycline (Tygacil): the first in the glycycline class of antibiotics. *Pharmacology notes: Baylor University Medical Center Proceedings,* 19, 155-161.

Grow, G. O. (1996). Teaching learners to be self-directed. *Adult Education Quarterly,* 41(3), 125-149.

Hawkey, P. M., Birkenhead, D., Kerr, K. G., Newton, K. E., & Hyde, W. A. (1993). Effect of divalent cations in bacteriological media in the susceptibility of *Xanthomonas maltophilia* to imipenem with special reference to zinc ions. *Journal of Antimicrobial Chemotherapy,* 31, 47-55.

Hirotoshi, M., Grubb, B. R., Tarran, R., Randell, S. H., Gatzy, J. T., Davis, C. W., & Boucher, R. C. (1998). Evidence for pericilliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell,* 95, 1005-1015.

Hutchinson, G. R., Parker, S., Pryor, J. A., Duncan-Skingle, F., Hoffman, P. N., Hodson, M. E., Kaufmann, M. E., & Pitt, T. L. (1996). Home-use nebulizers: a potential

primary source of *Burkholderia cepacia* and other colistin resistant Gram-negative bacteria in patients with cystic fibrosis. *Journal of Clinical Microbiology,* 34(3), 584-587.

Holden, M. T., Seth-Smith, H. M, Crossman, L. C, Sebaihia, M, Bentley, S. D, Cerdeño-Tárraga, A. M, Thomson, N. R, Bason, N, Quail, M. A, Sharp, S, Cherevach, I, Churcher, C, Goodhead, I, Hauser, H, Holroyd, N, Mungall, K, Scott, P, Walker, D, White, B, Rose, H, Iversen, P, Mil-Homens, D, Rocha, E. P., Fialho, A. M, Baldwin, A, Dowson, C, Barrell, B. G, Govan, J. R, Vandamme, P, Hart, C. A, Mahenthiralingam E, Parkhill, J. (2009) The genome of Burkholderia cenocepacia J2315, an epidemic pathogen of cystic fibrosis patients. *Journal of Bacteriology*. 191(1):261-77

Igra-Siegman, Y., Chmel, H., & Cobbs, C. (1980). Clinical and laboratory characteristics of *Achromobacter xylosoxidans* infection. *Journal of Clinical Microbiology,* 11(2), 141-145.

Inglis, T. J. I., Rodrigues, F., Rigby, P., Norton, R., & Currie, B. J. (2004). Comparison of the susceptibilities of *Burkholderia pseudomallei* to meropenem and ceftazidime by conventional and intracellular methods. *Antimicrobial Agents and Chemotherapy,* 48(8), 2999-3005.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P., & Levison, H. (1984). *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *Journal of Pediatrics,* 104, 206-210.

Jakobi, M., Winkelmann, G., Kaiser, D., Kempter, C., Jung, G., Berg, G., & Bahl, H. (1996). Maltophilin: a new antifungal compound produced by *Stenotrophomonas maltophilia* R3089. *Journal of Antibiotics*, 49, 1101-1104.

Johns, C. (2000). *Becoming a reflective practitioner – a holistic approach to clinical nursing, practice development and clinical supervision*. Oxford: Blackwell Science.

Jones, R. N., M. J. Ferraro, L. B. Reller, P. C. Schreckenberger, J. M. Swenson, and H. S. Sader. (2007). Multicenter studies of tigecycline disk diffusion susceptibility results for *Acinetobacter* spp. Journal of Clinical Microbiology. 45**:**227-230.

Joris, B., Galleni, M., Frere, M., & Labia, R. (1994). Analysis of the pen A gene of *Pseudomonas cepacia* 249. *Antimicrobial Agents and Chemotherapy,* 38, 407-408 (letter).

Kasbekar, N. (2006). "Tigecycline: a new glycylcycline antimicrobial agent.". *American Journal Health Sysemst and Pharmacology* 63 (13): 1235–43.

Keays T., Ferris W., Vandemheen K. L., Chan F., Yeung S. W., Mah T. F., Ramotar K., Saginur R., & Aaron S. (2009). A retrospective analysis of biofilm antibiotic susceptibility testing: A better predictor of clinical response in cystic fibrosis exacerbations. *Journal of Cystic Fibrosis*, 8, 122-127.

Kurlandsky L. E., & Fader R. C. (2000). *In-vitro* activity of minocycline against respiratory pathogens from patients with cystic fibrosis. *Pediatric Pulmonology* 29, 210-212.

Lekkas A., Gyi K. M., & Hodson M. E. (2006). Temocillin in the treatment of *Burkholderia cepacia* infection in cystic fibrosis. *Journal of Cystic Fibrosis*, 5, 121-124.

Li, J., Nation R. L., Milne R. W., Turnidge J. D., & Coulthard K. (2005). Evaluation of colistin as an agent against multi-resistant Gram negative bacteria. *International Journal of Antimicrobial Agents.* 25, 11-25.

LiPuma J. J. (2005) Update on the *Burkholderia cepacia* complex. *Current Opinion in Pulmonary Medicine*, 11 (6) 528-533

LiPuma J. J., Dasen S. E., Nielson D. W., Stern R. C., & Stull T. L. (1990). Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis. *The Lancet*, 336, 1094-1096.

LiPUma J. .J, Fisher M. C., Dasen S. E., Mortensen J. E., & Stull T. L. (1991). Ribotype stability of serial pulmonary isolates of *Pseudomonas capacia*. *Journal of Infectious Diseases*, 164, 133-136.

Liou T. G., Adler F. R., Cahill B. C., Fitzsimmons S. C., Huang D., & Hibbs J. R. (2005). Use of lung transplantation survival models to refine patient selection in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 171, 1053-1059.

Livermore D. M. (2005). Tigecycline: what is it, and where should it be used? *Journal of Antimicrobial Chemotherapy*, 56, 611-614.

Livermore, D. M., Tulkens, P. M. (2009). "Temocillin revived". *Journal of Antimicrobial Chemotherapy* 63 (2): 243–5.

Lyczak J. B., Cannon C. L., & Pier G. B. (2002). Lung infections associated with cystic fibrosis. *Clinical Microbiology Reviews*, 15, 194-222.

Mahenthiralingam E, Baldwin A, Vandamme P.(2002). *Burkholderia cepacia* complex infection in patients with cystic fibrosis. *Journal of Medical Microbiology*; 51: 533-538

Mahenthiralingam E., Campbell M. E., Henry D. A., & Speert D. P. (1996). Epidemiology of *Bukholderia cepacia* Infection in patients with cystic fibrosis: Analysis by

randomly amplified polymorphic DNA fingerprinting. *Journal of Clinical Microbiology*, 39(12), 2914-2920.

Mahenthiralingam E., Simpson D. A., & Speert D. P. (1997). Identification and characterisation of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. *Journal of Clinical Microbiology* 35(4), 808-816.

Mall M., Grubb B. R., Harkema J. R., O'Neal W. K., & Boucher R. C. (2004). Increased airway epithelial Na absorption produces cystic fibrosis like lung disease in mice. *Nature Medicine* 10, 487-493.

Manno G., Ugolotti E., Belli M. L., Fenu, M.L., Romano, L., & Cruciani M. (2003). Use of the E test to assess synergy of antibiotic combinations against isolates of *Burkholderia cepacia* complex from patients with cystic fibrosis. *European Journal of Clinical Microbiology and Infectious Diseases,* 22(1), 28-34.

Mariscal D., Bosque M., Pomares X., Ferreros P., Garcia I,. Monton C., & Gallego M. (2006). Combination bactericidal antibiotic testing to multi-resistant strains of *Pseudomonas aeruginosa, cepacia* and *Stenotrophomonas maltophilia* isolated from patients with cystic fibrosis (CF). *Chest* 130(4), 139(supplement).

McDowell A., Mahenthiralingam E., Dunbar K. E. A., Moore J. E., Crowe M., & Elborn J. S. (2004). Epidemiology of *Burkholderia cepacia* complex species recovered from cystic fibrosis patients: issues related to patient segregation. *Journal of Medical Microbiology,* 53, 663-668.

Milatovic, D., Schmitz, F. J., Verhoef, J., Fluit A. C. (2003). Activities of the glycylcycline tigecycline (GAR-936) against 1,924 recent European clinical bacterial isolates. *Antimicrobial Agents and Chemotherapy*. 47:400–404.

Millar, F. A., Symmonds, F. J., Hodson, M. E. (2009). Trends in pathogens colonising the respiratory tract of adult patients with cystic fibrosis, 1985–2005. *Journal of Cystic Fibrosis*. 8 (6): 386-391

Moellering R. C. (1979). Antimicrobial synergism – an elusive concept. *Journal of Infectious Disease*, 140, 639-641.

Moore H. B., & Pickett M. J. (1960). Organisms resembling *Alcaligenes faecalis*. *Canadian Journal of Microbiology*, 6, 43-52.

O'Riordan S. M., Robinson P. D., Donaghue K. C., & Moran A. M. (2008). Management of cystic fibrosis related diabetes. *Pediatric Diabetes*, 9, 338-344.

O'Sullivan B., & Freedman S. (2009). Cystic Fibrosis. *The Lancet*, 373, 1891-1904,

Orhan G., Bayram A,. Zer Y., & Balci I. (2005). Synergy tests by E-test and checkerboard methods of antimicrobial combinations against *Brucella melitensis*. *Journal of Clinical Microbiology,* 43(1), 140-143.

Palleroni N. J., & Bradbury J. F. (1993). *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings *et al* 1983. *International Journal of Systematic Bacteriology*, 43, 606-609.

Papapetropolou M., Rodopolou G., Gionnoulaki E., & Stergiopoulos P. (1994). Effect of temperature on antimicrobial susceptibilities of *Pseudomonas* species isolated from drinking water. *Journal of Chemotherapy*, 6, 404-407.

Pankey G., Ashcraft D., & Patel N. (2005). *In-vitro* synergy of daptomycin plus rifampin against *Enterococcus faecium* resistant to both linezolid and vancomycin. *Antimicrobial Agents and Chemotherapy,* 49(12), 5166-5168.

Pankey, G., & Ashcraft D. (2005). *In Vitro* Synergy of Ciprofloxacin and Gatifloxacin against Ciprofloxacin-Resistant *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy,* 49, (7), 2959–2964

Payne D. J., Bateson J. H., Gasson B. C., Proctor D., Khushi T., Farmer T. H., Tolson D. A., Bell D., Skett P. W., Marshall A. C., Reid R., Ghosez L., Combret Y., & Barchand-Brynaert J. (1997). Inhibition of metallo-beta lactamases by a series of mercaptoacetic acid thiol ester derivatives. *Antimicrobial Agents and Chemotherapy*, 41, 135-140.

Petersen P. J., Labthavikul P., Jones C. H., & Bradford P. A. (2006). *In vitro* antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time kill kinetic analysis. *Journal of Antimicrobial Chemotherapy,* 57, 573-576.

Peterson L. R., & Shanholtzer C. J. (1992). Tests for bactericidal effects of antimicrobial agents: technical performance and clinical relevance. *Clinical Microbiology Reviews,* 5(4), 420-432.

Poirel L., Rodriguez-Martinez J. M., Plesiat P., & Nordmann P. (2009). Naturally occurring class A β-lactamases from the *Burkholderia cepacia* complex. *Antimicrobial Agents and Chemotherapy,* 53(3), 876-882.

Principe L., D'Arezzo S., Capone A., Petrosillo N., & Visca P. (2009). *In vitro* activity of tigecycline in combination with various antimicrobials against multidrug resistant *Acinetobacter baumannii*. *Annals of Clinical Microbiology and Antimicrobials,* 8(18), 18.

Randell S. H., & Boucher R. C. (2006). Effective mucus clearance is essential for respiratory health. *American Journal of Respiratory Cell and Molecular Biology,* 35, 20-28.

H. P. Rang, M. M. Dale., & J. M. Ritter (Eds.). (1995). *Pharmacology* (3rd Ed.). pp 718-743. London: Churchill Livingstone.

Ratjen, F. A. (2009). Cystic Fibrosis: Pathogenesis and Future Treatment Strategies. *Respiratory Care*, 54(5), 595-605.

Reik R., Spilker T., & LiPuma J. (2005). Distribution of *Burkholderia cepacia* complex species among isolates recovered from persons with or without cystic fibrosis. *Journal of Clinical Microbiology,* 43(6), 2926-2928.

Report of the UK cystic fibrosis trust infection control group, Cystic Fibrosis Trust. (2004). The *Burkholderia Cepacia* complex – Suggestions for prevention and infection control.

http://www.cftrust.org.uk/aboutcf/publications/consensusdoc/C_Burkholderia_cepacia_Sep_2004.pdf (accessed 15/9/10)

Rogers G. B., Carroll M. P., Serisier D. J., Hockey P. M., Jones G., & Bruce K. D. (2004). Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymporhism profiling. *Journal of Clinical Microbiology,* 42, 5176-5183.

Rogers G. B., Carroll M. P., Serisier D. J., Hockey P. M., Kehagia V., Jones G. R., & Bruce K. D. (2005). Bacterial activity in cystic fibrosis lung infections. *Respiratory Research,* 6, 49-60.

Ronne Hansen C, Pressler T, Hoiby N, Gormsen, N. (2006). Chronic infection with *Achromobacter xylosoxidans* in cystic fibrosis patients: a retrospective case-control study. *Journal of Cystic Fibrosis* 5: 245-251

Sachet D.L. , Rosenberg W. M. C., Muir-Gray J. A., Haynes R. B., & Richardson S. (1996). Evidence Based Practice – what it is and what it isn't. *British Medical Journal*, 312, 71-72.

Saiman L. (2007). Clinical utility of synergy testing for multidrug-resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis "the motion for". *Paediatric Respiratory Reviews,* 8, 249-255.

Saiman L., & Siegel J. (2004). Infection control in cystic fibrosis. *Clinical Microbiology Reveiws,* 17(1), 57-71.

Saiman L., Chen Y., Tabibi S., San Gabriel P., Zhou J., Liu Z., Lai L., & Whittier S. (2001). Identification and antimicrobial susceptibility of *Alcaligenes xylosoxidans* isolated from patients with cystic fibrosis. *Journal of Clinical Microbiology,* 39(11), 3942-3945.

Saiman L., Chen Y., San Gabriel P., & Knirsch, C. (2002). Synergistic activities of macrolide antibiotics against *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, and *Alcaligines xylosoxidans* isolated from patients with Cystic Fibrosis. *Antimicrobial Agents and Chemotherapy*, 46(4), 1105-1107.

San Gabriel P., Zhou J., Tabibi S., Chen Y., Trauzzi M., & Saiman L. (2004). Antimicrobial susceptibility and synergy studies of *Stenotrophomonas maltophilia* isolates from patients with cystic fibrosis. *Antimicrobial Agents and Chemotherapy,* 48(1), 168-171.

Schon, D. (1987). *Educating the reflective practitioner*. San Francisco: Jossey-Bass.

Sharma G. D., Tosi M. F., Stern R. C., & Davis P. B. (1995). Progression of pulmonary disease after disappearance of *Pseudomonas* in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 152, 169-173.

Shawar, R. M., MacLeod, D. L., Garber, R. L., Burns, J. L., Stapp, J. R., Clausen, C. R., Tanaka, S. K. (1999) Activities of tobramycin and six other antibiotics against *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrobial Agents and Chemotherapy*; 43: 2877-2880.

Siebor, E. (2006). VEB-1 in *Achromobacter xylosoxidans* form cystic fibrosis patient, France. *Emerging Infectious Diseases,* 12(11), 1737-1739.

Smith A. L., Doershuk C., Goldman D., Gore E., Hilman B., Marks M., Moss R., Ramsey B., Redding G., Rubio T., Williams-Warren J,. Wilmott R., & Wilson H. D. (1999). Comparison of beta-lactam alone versus beta-lactam and an aminoglycoside for pulmonary exacerbation in cystic fibrosis. *Journal of Paediatrics*, 134, 413-421.

Smith A. L., Standley B., Mayer-Hamblett N., Ramsey B., & Burns J. L. (2003). Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. *Chest*, 123, 1495-1502.

Spear J. B., Fuhrer J., & Kirby B. D. (1987). *Achromobacter xylosoxidans* (*Alcaligenes xylosoxidans* subsp. x*ylosoxidans*) bacteremia associated with a well-water source: Case report and review of the literature. *Journal of Clinical Microbiology*, 26(3), 598-599.

Speert D. P., Henry D., Vandamme P., Corey M., & Mahenthiralingam E. (2002). Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada. *Emerging Infectious Diseases,* 8(2), 181-187.

Steinbach, S., Sun, L., Jiang, R. Z., Flume, P., Gilligan, P., Egan, T. M., Goldstein, R. (1994) Transmissibility of *Pseudomonas cepacia* infection in clinic patients and lungtransplant recipients with cystic fibrosis. *New England Journal of Medicine.* 331: 981-987.

Tarran R., Grubb B. R., Parsons D., Picher M., Hirsh A. J., Davis C. W., & Boucher R. C. (2001). The CF Salt Controversy: *In vivo* observations and therapeutic approaches. *Molecular Cell*, 8, 149-158.

Taylor R., Gaya H., & Hodson M. (1992). Temocillin and cystic fibrosis: outcome of intravenous administration in patients with *Pseudomonas cepacia*. *Journal of Antimicrobial Chemotherapy,* 29, 341-344.

Teekman B. (2000). Exploring reflective thinking in nursing practice. *Journal of Advanced Nursing,* 31(5), 1125-1135.

Tenover F. C., Arbeit R. D., Goering R. V., Mickelsen P. A., Murray B. E., Persing D. H., & Swaminathan B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed field gel electrophoresis: Criteria for bacterial strain typing. *Journal of Clinical Microbiology,* 33(9), 2233-2239.

Tilton R. C., Steingrimsson O., & Ryan R. W. (1978). Susceptibilities of *Pseudomonas* species to tetracycline, minocycline, gentamicin and tobramycin. *American Journal of Clinical Pathology,* 69, 410-413.

Todd A., Palfreyman S., & Burke L. (2004). Evidence Based Practice is a time of opportunity for nusing. *British Journal of Nursing*, 13(4), 211-216.

Tyler S. D., Strathdee C. A., Rozee K. R., & Johnson W. M. (1995). Oligonucleotide primers designed to differentiate pathogenic *Pseudomonads* on the basis of sequencing of genes coding for 16S-22S rRNA internal transcribed spacers. *Clinical and Diagnostic Laboratory Immunology*, 2, 448-453

Valerius N. H., Koch C., & Hoiby N. (1991). Prevention of chronic *Pseudomonas aeruginosa* colonisation in cystic fibrosis by early treatment. *The Lancet*; 338, 725-672.

Valdezate S., Vindel A., Martin-Davila P., Del Saz B.S., Baquero F., & Canton R. (2004). High genetic diversity among *Stenotrophomonas maltophilia* strains despite their originating at a single hospital. *Journal of Clinical Microbiology*, 42(2), 693-699.

Vanlaere E., LiPuma J. J., Baldwin A., Henry D., De Brandt E., Mahenthiralingam E., Speert D., Dowson D., & Vandamme P. (2008). *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex. *Journal of Systemic and Evolutionary Microbiology*, 58, 1580-1590.

Vanlaere E., Baldwin A., Gevers D., Henry D., De Brandt E., LiPuma J. J., Mehanthiralingham E., Speert D. P., Dowson C., & Vandamme P. (2009). Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species,

Burkholderia contaminans sp. nov. and *Burkholderia lata* sp. nov. *Journal of Systemic and Evolutionary Microbiology*, 59, 102-111.

Vartivarian S., Anaissie E., Bodey G., Sprigg H., & Rolston K. (1994). A changing pattern of susceptibility of *Xanthomonas maltophilia* to antimicrobial agents: implications for therapy. *Antimicrobial Agents and Chemotherapy*, 38(3), 624-627.

Walters S., & Mehta A. (2007). Epidemiology of cystic fibrosis. In M. Hodson, D. M. Geddes, & A. Bush (Eds.). *Cystic fibrosis*, 3rd edition (pp21-45). London: Edward Arnold Ltd.

Wheat P. F., Winstanley T. G., & Spencer R. C. (1985). Effect of temperature on antimicrobial susceptibilities of *Pseudomonas maltophilia*. *Journal of Clinical Pathology*, 38, 1055-1058.

White R. L., Burgess D. S., Manduru M., & Bosso J. A. (1996). Comparison of three different in vitro methods of detecting synergy: Time Kill, Checkerboard, and E-test. *Antimicrobial Agents and Chemotherapy*, 40(8), 1914-1918.

Wine J. J. (1999). The genesis of cystic fibrosis lung disease. *Journal of Clinical Investigation*, 103, 309-312.

Worlitzsch D., Tarran R., Ulrich M., Schwab U., Cekici A., Meyer K. C., Birrer P., Bellon G., Berger J., Weiss T., Botzenhart K., Yankaskas J. R., Randell S., Boucher R. C., Döring G. (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *Journal of Clinical Investigation*, 109(3), 317-325.

Yabuuchi E., & Ohyama A. (1971). *Achromobacter xylosoxidans* n.sp. from human ear discharge. *Japanese Journal of Microbiology*, 15, 477-481.

Yao J. D., Louie M., Louie L., Goodfellow J., & Simor A. E. (1995). Comparison of E-test and agar dilution for antimicrobial susceptibility testing of *Stenotrophomonas* (*Xanthomonas*) *maltophilia*. *Journal of Clinical Microbiology,* 33, 1428-1430.

Yi H., Srivivasan S., & Kim M. K. (2010). *Stenotrophomonas panacihumi* sp.*nov*., isolated from soil of a ginseng field. *Journal of Microbiology,* 48(1), 30-35.

Zhang L., Li X., & Poole K. (2001). Fluoroquinolone susceptibilities of efflux-mediated multidrug-resistant *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*. *Journal of Antimicrobial Chemotherapy,* 48, 549-552.

Appendix 1

Below is the response from the Leeds Research Ethics Committee that was received prior to starting practical work on this project.

Leeds (West) Research Ethics Committee

A/B Floor, Old Site

Leeds General Infirmary

Great George Street

Leeds

LS1 3EX

Telephone: 0113 3923181

Facsimile: 0113 392 2863

19 March 2008

Dr Miles Denton

Consultant Microbiologist

Leeds Teaching Hospitals Trust

Department of Microbiology

Leeds General Infirmary

Leeds

LS1 3EX

UK

Dear Dr Denton

Full title of study:	IN VITRO SUSCEPTIBILITY TESTING OF BURKHOLDERIA CEPACIA COMPLEX AND OTHER MULTI-RESISTANT NON-FERMENTATIVE GRAM NEGATIVE BACILLI (GNBs) ISOLATED FROM PEOPLE WITH CYSTIC FIBROSIS
REC reference number:	08/H1307/42

The Research Ethics Committee reviewed the above application at the meeting held on 14 March 2008.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to submit the Site-Specific Information Form to any Research Ethics Committee. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*	
Application	1		
Investigator CV	J Kenning		
Investigator CV	G Mills		
Investigator CV	M Denton		
Protocol	1		

R&D approval

You should arrange for the R&D office at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research at a NHS site must obtain final approval from the R&D office before commencing any research procedures.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.

b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

With the Committee's best wishes for the success of this project

Yours sincerely

Laura Sawiuk

REC Co-ordinator

On Behalf of

Dr Michael Rivlin

Vice Chair

Email: laura.sawiuk@leedsth.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
Standard approval conditions

Copy to: *Dr Derek Norfolk, Leeds Teaching Hospitals NHS Trust*

Leeds (West) Research Ethics Committee

Attendance at Committee meeting on 14 March 2008

Committee Members:

Name	*Profession*	*Present*	*Notes*	
Miss Petra Bijsterveld	BHF Research Nurse	Yes		
Professor Howard Bird	Consultant Rheumatologist	Yes		
Dr Michael Blackburn	Consultant Paediatric Cardiologist	Yes		
Mrs Rhona Bratt	Lay Member	Yes		
Mrs Sheila E. Fisher	Senior Lecturer / Hon Consultant in Oral & Maxillofacial Surgery	Yes		
Dr Stella Kwan	Senior Lecturer in Dental Public Health	Yes		
Mr Peter Margerison	Lay Member	Yes		
Miss Eve Miles	Lay Member	Yes		
Dr Wendy Neil	Consultant Psychiatrist	No		
Dr Vera Neumann	Consultant in Rehabilitation Medicine	Yes		
Dr Michael Rivlin	Lay Member, Medical Ethics Lecturer	Yes		
Mr Andrew Scally	Statistician	No		
Dr Ken Shenderey	General Practitioner	Yes		

Mr Jon Silcock	Lecturer in Pharmacy	No		
Revd. Chris Swift	Lay Member, Chaplain	Yes		
Mr Daniel Williams	Lay + Member	Yes		

Also in attendance:

Name	*Position (or reason for attending)*	
Miss Laura Sawiuk	REC Co-ordinator	

Written comments received from:

Name	*Position*	
Mr Andrew Scally	Statistician	
Mr Jon Silcock	Lecturer in Pharmacy	

Appendix 2

Below is the abstract and draft of the journal article prepared with Dr Denton for publication and presentation both in the *Journal of Antimicrobial Chemotherapy*, and *Journal of Cystic Fibrosis*. This abstract was also presented with a poster at the ECCMID conference in Helsinki.

The in vitro activity of tigecycline and other antimicrobial agents against *Burkholderia cepacia* complex and other cystic fibrosis-associated Gram negative bacteria.

John Kenning[1], Steven Conway[2] and Miles Denton[1]*

[1]Department of Microbiology, Leeds General Infirmary, Great George Street, Leeds, LS1 3EX, United Kingdom

[2]Regional Adult and Paediatric Cystic Fibrosis Centres, St James's University Hospital, Leeds, LS9 7TF, United Kingdom

***Corresponding author**

Dr Miles Denton

Department of Microbiology

Leeds General Infirmary

Great George Street

Leeds

LS1 3EX

E-mail: miles.denton@leedsth.nhs.uk

Tel: + 44 113 392 2922

Fax: + 44 113 392 2696

Summary

Background: Options for treating *Burkholderia cepacia* complex (Bcc) and other multi-resistant Gram negative bacilli isolated from people with CF are limited. We assessed the in vitro activity of tigecycline and eleven other antimicrobial agents against a collection of these organisms

Methods: The collection comprised of 148 isolates of CF-associated Gram negative bacilli (36 *Burkholderia multivorans*, 20 *Burkholderia cenocepacia*, 7 other members of the Bcc, 48 *Stenotrophomonas maltophilia*, 20 *Achromobacter xylosoxidans*, 17 other miscellaneous CF-associated Gram negative bacilli). Minimum inhibitory concentrations of tigecycline and eleven other antimicrobials for each isolate were determined using Etest. Synergy between tigecycline and each of eight other antimicrobials was determined using an Etest overlay method.

Results: Tigecycline showed poor in vitro activity versus all members of the Bcc, with only 10% and 3% of *B. cenocepacia* and *B. multivorans* susceptible, respectively. Conversely minocycline showed good activity against these species, with 85% and 92% of isolates being susceptible. Tigecycline showed good activity against *A. xyloxidans* and *S. maltophilia* with 85% and 77% of isolates being susceptible, respectively. Tigecycline in combination with other agents mostly resulted in indifference.

Conclusions: Although the in vitro activity of tigecycline against many of these difficult to treat species appeared variable it remains unclear how these results correlate with clinical outcomes. Further clinical studies are warranted.

Introduction

Cystic fibrosis (CF) is a serious genetically inherited disease affecting around 1 in 2000 live births particularly those of Caucasian background. People with CF are prone to recurrent respiratory tract infections, most commonly with *Pseudomonas aeruginosa*. However other multi-resistant Gram negative non-fermentative bacteria are frequently isolated from people with CF, including *Burkholderia cepacia* complex (Bcc), *Stenotrophomonas maltophilia* and others.[1] Some members of the Bcc, most commonly *Burkholderia cenocepacia* IIIA are a major cause of rapid deterioration, sepsis and death ("cepacia syndrome") in CF.[2]

Therapy for many of these multi-resistant Gram negative non-fermentative bacteria is compromised by resistance to many of the available antibiotics.[3] Current options are therefore limited and are further compromised by frequent allergies to antibiotics, particularly beta-lactams and concerns regarding toxicity.

Minocycline (a tetracycline) has been shown to have useful activity against many of the multi-resistant GNBs isolated from people with CF.[4,5] However it is only available orally, which may not be appropriate in all clinical cases. If tigecycline, a novel injectable glycylcycline antibiotic derived from minocycline, was shown to have reliable activity against these difficult CF-associated pathogens it would enhance the options available for treatment. This study assesses the in vitro activity of tigecycline and other commonly used antibiotics against Bcc and other CF-associated Gram negative bacteria.

Methods

Isolate collection

The following collection of isolates was used in the study: 36 *Burkholderia multivorans* (33 clinical, 3 laboratory type strains); 20 *B. cenocepacia* (16 clinical, 4 laboratory type strains); seven other Bcc members comprising *Burkholderia cepacia* (1 type strain), *Burkholderia stabilis* (1 type, 1 clinical), *Burkholderia vietnamiensis* (1 type, 1 clinical) and *Burkholderia pyrrocinia* (1 type); 20 *Achromobacter xylosoxidans* (all clinical); 48 *S. maltophilia* (47 clinical, 1 type); 17 other assorted CF-associated Gram-negative bacilli, including *Burkholderia gladioli*, *Pandoraea* species and *Ralstonia* species (9 clinical, 8 type). All clinical isolates had been collected from patients attending the Regional Paediatric and Adult CF Units in Leeds and identified using conventional laboratory methods. Identification of all members of the Bcc and other isolates had been confirmed using molecular methods after submission to a reference laboratory (Centre for Infections, Health Protection Agency, Colindale, London, UK). Type strains had been provided by EuroCare CF (University of Ghent, Belgium). Isolates had been stored in 15% glycerol broth at -70^{o}C until required.

Genotyping

All isolates were subjected to pulsed field gel electrophoresis (PFGE) to identify multiple isolates of the same strain. The method used was identical to one previously published with a single modification that *Spe*I was used in place of *Xba*I as the restriction endonuclease.[6]

Determination of minimum inhibitory concentrations (MICs) using Etest

The MIC of eleven agents (aztreonam, amikacin, ceftazidime, ciprofloxacin, colistin, meropenem, minocycline, piperacillin-tazobactam, temocillin, tigecycline, tobramycin) was determined using Etest (AB Biodisk, Solna, Sweden). Etests were performed on Mueller-Hinton II agar (MHA) plates according to the manufacturer's instructions. Plates were incubated overnight in air at 37^{o}C, except isolates of *S. maltophilia*, which were incubated at 30^{o}C. Each isolate was tested in duplicate. MICs in between twofold dilutions were rounded up to the next twofold dilution for purposes of comparison. None of the tested isolates have species-specific breakpoints available in recognised guidelines for the agents tested. Isolates were called susceptible, intermediate or resistant on the basis of British Society for Antimicrobial Chemotherapy (BSAC) breakpoints used for *P. aeruginosa*, except for tigecycline and temocillin (published BSAC breakpoints for Enterobacteriaceae were used), and minocycline (the Clinical and Laboratory Standards Institute breakpoint for non-Enterobactericeae was used).

Synergy testing using Etest

Synergy testing between tigecycline in combination with one of eight other agents (aztreonam, ceftazidime, ciprofloxacin, colistin, meropenem, piperacilllin-tazobactam, temocillin, tobramycin) was performed in duplicate, with the summation fractional inhibitory concentration (FIC) calculated for each set of MICs, using the Etest method reported by Pankey and Ashcraft.[7] The inoculum and streaked MHA plates for each isolate were prepared the same as for Etest MICs. An Etest strip of drug *a* was applied to a MHA plate and then removed after 1h at room temperature. Using an Etest applicator, a strip for drug *b* was placed over the area of the previously removed drug *a* strip. The resulting combination ellipses were read after a further 20 h of incubation at 35°C, except for *S. maltophilia*, which was incubated at 30^{o}C.

The effect of tigecycline in combination was assessed by calculating the FIC for each antibiotic in each combination. The following formulae were used to calculate the ∑FIC:

FIC of drug *a* = (MIC of drug *a* in combination)/(MIC of drug *a* alone)

FIC of drug *b* = (MIC of drug *b* in combination)/(MIC of drug *b* alone)

∑FIC = FIC of drug *a* + FIC of drug *b*.

Synergy was defined by a ∑FIC of ≤ 0.5. Antagonism was defined by a ∑FIC of

> 4. Interactions represented by a ∑FIC of > 0.5 but ≤ 4 were termed indifferent.

Results and discussion

The results of MIC determination and synergy testing are shown in tables 1 and 2 respectively. Tigecycline was poorly active against the Bcc, with only 10%, 3% and 14% of *B. cenocepacia*, *B. multivorans* and other Bcc isolates being susceptible, respectively. By comparison the activity of minocycline was much greater, with 85%, 92% and 86% of isolates being susceptible. The beta-lactams ceftazidime and meropenem were more active than tigecycline against the Bcc, with 60% and 50% of *B. cenocepacia* and 58% and 39% of *B. multivorans* being susceptible, respectively. Temocillin was active against 40% of *B. cenocepacia* and 44% of *B. multivorans* isolates

The activity of tigecycline against *A. xylosoxidans* and *S. maltophilia* was much more reliable, with 85% and 77% of isolates being susceptible. Minocycline activity was similar, with 80% and 96% susceptibility for the same isolates. Piperacillin-tazobactam was the only beta-lactam with comparable activity versus *A. xylosoxidans* with 85% susceptibility. The most active beta-lactam against *S. maltophilia* was ceftazidime but only 48% of isolates appeared susceptible.

As expected, colistin had no activity against the Bcc. Activity against *A. xylosoxidans* and *S. maltophilia* was moderate. The activity of ciprofloxacin against *B. cenocepacia*, *B. multivorans*, *A. xylosoxidans* and *S. maltophilia* was poor throughout. Aminoglycosides also demonstrated poor activity against all species groups.

The combination of tigecycline with other agents usually resulted in indifference against tested isolates. Antagonism was rarely encountered, except when used in combination with colistin. Conversely the most synergistic combination was tigecycline with colistin against *A. xylosoxidans*, with synergy demonstrated against 40% of isolates.

The activity of tigecycline against tested isolates was therefore variable and largely showed indifference in combination with other agents. However, the correlation between in vitro susceptibility test results and clinical outcomes for these species is unknown. Species-specific breakpoints are not available at present and therefore definitions of susceptible and resistant remain arbitrary. This is further complicated in the setting of CF where infection with organisms such as the Bcc are often chronic rather than acute. Correlation between in vitro susceptibility testing results and clinical outcomes in chronic *P. aeruginosa* infections in CF is well recognised as poor and patients frequently respond to therapy even when their isolates are reported as resistant to the agents used.[8] Clinical data regarding the therapeutic utility of tigecycline or minocycline in treating Bcc, *A. xylosoxidans* or *S. maltophilia* infection in CF is currently lacking and needs further study. This is particularly important as therapeutic options are often limited by drug allergy, particularly to beta-lactams, and toxicity concerns, most commonly with aminoglycosides.

The optimum method of susceptibility testing of these isolates from people with CF has been a subject of much debate. Although selection of treatment regimens based on the results of in vitro synergy testing has been advocated, a double-blind randomised controlled trial failed to demonstrate any benefit for regimens showing synergy *in vitro* in comparison to those that do not.[9] More recently, novel approaches to susceptibility testing, such biofilm and stationary-phase models,[10] have been reported but none as yet have been shown to be more predictive of clinical outcomes in comparison to traditional susceptibility testing methods.

The observation that tigecycline appears significantly less active *in vitro* than minocycline against members of the Bcc is interesting and warrants further investigation. Possible explanations are that tigecycline uptake into Bcc cells is via different channels to that of minocycline, that Bcc possesses as yet unknown efflux mechanisms that preferentially work against tigecycline compared to minocycline, or that technical variables (e.g. cationic content of media) during in vitro testing are exerting an influence.

Genotyping by PFGE revealed that little clustering of isolates for most species (data not shown). This was not surprising as epidemiological studies of *B. multivorans, A. xylosoxidans* and *S. maltophilia* in CF have consistently found most patients have unique strains, suggesting independent acquisition from presumed environmental sources. Several (JOHN – need to see PFGE gel to get exact numbers) of the *B. cenocepacia* isolates clustered with the ET-12 epidemic strain. However, susceptibility of these isolates to tigecycline and other agents was variable, suggesting differential expression of resistance. This is also worthy of further investigation.

In summary, the in vitro activity of tigecycline against Bcc was poor but promising against *A. xylosoxidans* and *S. maltophilia*. Further clinical studies are required to ascertain how these in vitro findings correlate with clinical outcomes when used to treat people with CF.

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Ethical approval

The study was approved by the Leeds (West) Research Ethics Committee, REC reference 08/H1307/5.

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Transparency declaration

Nothing to declare.

References:

Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. Clin Microbiol Rev 2002; 15: 194-222.

Isles A, Maclusky I, Corey M, et al. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. J Pediatr 1984; 104: 206-10.

Conway SP, Brownlee KG, Denton M, Peckham DG. Antibiotic treatment of multi-drug-resistant organisms in cystic fibrosis. Am J Respir Med 2003; 2: 321-32.

Kurlandsky LE, Fader RC. In vitro activity of minocycline against respiratory pathogens from patients with cystic fibrosis. Pediatr Pulmonol 2000; 29: 210-2.

Saiman L, Chen Y, Tabibi S, et al. Identification and antimicrobial susceptibility of *Alcaligenes xylosoxidans* isolated from patients with cystic fibrosis. J Clin Microbiol 2001; 39: 3942-5.

Denton M, Todd NJ, Kerr KG, et al. Molecular epidemiology of *Stenotrophomonas maltophilia* isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. J Clin Microbiol 1998; 36: 1953-8.

Pankey GA, Ashcraft DS. In vitro synergy of ciprofloxacin and gatifloxacin against ciprofloxacin- resistant *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2005; 49: 2959-64.

Smith AL, Fiel SB, Mayer-Hamblett N, et al. Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. Chest 2003; 123: 1495-502.

Aaron SD, Vandenheem KL, Ferris W, et al. Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multi-resistant bacteria: a randomised, double-blind controlled clinical trial. Lancet 2005; 366: 463-71.

Peeters E, Nelis HJ, Coenye T. In vitro activity of ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin and trimethoprim/sulfamethoxazole against planktonic and sessile*Burkholderia cepacia* complex bacteria. J Antimicrob Chemother 2009; 64: 801-9.

Combination testing of multidrug-resistant cystic fibrosis isolates of Pseudomonas aeruginosa: use of a new parameter, the susceptible breakpoint index.

Milne KE, Gould IM.

J Antimicrob Chemother. 2010 Jan;65(1):82-90. Epub

Accuracy and cost of antibiotic susceptibility testing of mixed morphotypes of Pseudomonas aeruginosa.

Morlin GL, Hedges DL, Smith AL, Burns JL.

J Clin Microbiol. 1994 Apr;32(4):1027-30.

Appendix 3

Declaration of work for the award of Doctor of Biomedical Science:

As registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.